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Full Paper

The effect of culturing temperature on the growth of the most dominant bacterial species of human gut microbiota and harmful bacterial species

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In recent years, the gut microbiota has attracted attention due to reported associations with various diseases and health conditions. Gut bacteria have been constantly cultured at 37°C, potentially limiting the understanding of the interaction between them and the host. However, the most dominant human gut microbial species have not been extensively cultured at temperatures other than 37°C. In this study, we analyzed the effects of various culturing temperatures on the growth of the 51 most dominant commensal species as well as 3 harmful bacteria, including *Clostridium perfringens*, a food poisoning bacterium, in the human intestine. The results showed that the growth of predominant gut microbes varied minimally at body temperatures conducive to human survival but that the growth of several bacteria involved in butyrate production in the intestinal lumen was repressed at temperatures other than 37°C. When cultured at 50°C, the growth of *C. perfringens* was less inhibited than that of other bacterial species. In addition, the growth of some gut bacteria was unaffected by a body temperature range that was not suitable for human survival.

Key words: gut microbiota, culturing temperature, clostridium perfringens

INTRODUCTION

Animals possess complex microbial communities within their intestines, and an intricate interplay exists between the gut microbiota and physical illnesses, mental disorders, and health conditions.

In the context of physical disease, *Bacteroides* spp. proliferate in the intestinal tract of obese individuals who achieve weight loss by restricting fat or carbohydrates [1]. Moreover, the alteration of gut microbiota by artificial sweeteners is associated with glucose intolerance in both humans and mice [2], and the gut microbiota of wild mice improves resistance to disease, such as influenza virus infection and colorectal tumorigenesis [3].

In the context of mental disorders, administering the human commensal bacterium *Bacteroides fragilis* suppressed autism-like behaviors in a mouse model of autism spectrum disorder (ASD) [4]. Moreover, administering the gut microbiota of individuals with ASD altered the behavior of mice to autistic-like [5]. Furthermore, the social behavior phenotype of the *Cntnap2*$^{-/-}$ mouse, a neurodevelopmental disorder model, was influenced by the gut microbiota [6], and individuals with a high prevalence of *Bacteroides* spp. in their guts are susceptible to depression [7].

In terms of health status, studies have shown that increasing levels of colonic polyamines, a metabolite of gut bacteria, improves cognition and extends lifespan [8, 9] and that motor function is regulated by the metabolites of gut bacteria, linking the gut microbiome to midbrain dopamine signaling via enteric sensory neurons [10].

The gut commensal microbiota includes bacteria such as *Clostridium perfringens*, which has long been considered a food poisoning bacterium; *Clostridioides difficile*, which is known as the causative bacterium of pseudomembranous colitis [8, 9]; and *Fusobacterium nucleatum*, which is known as a causative bacterium of periodontal disease, with reported implications in colorectal cancer invasion [10].

Among these bacteria, *C. perfringens* is a gram-positive anaerobic bacterium found in a variety of environments, including soil, sewage, livestock, and food. Toxins produced by the bacteria have been reported to cause diarrheal diseases and other food poisoning [11]. *C. perfringens* forms spores that can withstand

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cooking temperatures, so if cooked food is left to stand for a long time, it will germinate and form infectious bacterial colonies [12].

Thermostatic animals regulate their body temperature within a specific range, and humans are among the most adept at this regulation. For example, in a high-temperature environment, autonomic nervous system responses dilate skin blood vessels, releasing heat, and perspiration dissipates heat by evaporation, preventing a rise in body temperature [13]. This physiological mechanism enables humans to withstand elevated body temperatures up to 41°C; however, fatality ensues upon surpassing this critical threshold [14].

Conversely, hypothermia is a condition in which the body temperature drops below 35°C [15] and is associated with a high risk of serious illness and mortality [16]. The leading cause of death at low temperatures is cardiac arrest due to cooling of the heart. A drop in body temperature to below 32°C induces ventricular fibrillation, which increases cardiac arrest risk [16]. Therefore, a 5–6°C change in temperature from the normal body temperature can be fatal for humans.

A previous study that investigated the effects of acute cold-immersion restraint stress on intestinal injury and gut microbiota distribution reported that cold-immersion restraint decreases gut microbiota diversity in mice compared with that in its control groups. Specifically, aerobic and gram-negative bacteria notably increased following cold-immersion restraint, which was correlated with the extent of intestinal injury resulting from the cold-immersion restraint [17]. This finding suggests that some gut bacteria can grow at temperatures below 37°C, which alters the balance of the microbiota.

Another study demonstrated that an increase in body temperature to above 38°C enhances resistance to pathogenic viruses. This research reported that the gut microbiota, activated by the increased body temperature, elevated secondary bile acid levels in the body, thereby suppressing viral replication and the viral infection-associated inflammatory response [18]. This finding suggests that some gut bacteria can grow and actively metabolize at temperatures above 37°C.

Taken together, maintaining a constant culture temperature of 37°C for gut bacteria may limit our understanding of gut bacteria and their interactions with the host. However, no previous comprehensive study has cultured the most dominant species of human gut commensal microbiota at a temperature other than 37°C.

Thus, the effects of hypothermia and hyperthermia on the host are well known, but their effects on gut bacteria are not. This study will contribute to a deeper understanding of how small changes in temperature affect the human microbiota and its potential relevance to gastrointestinal pathology, based on the already known ecological characteristics of microorganisms and their interactions with the host. The impacts of microorganisms on host health are complex, and understanding how changes in culture temperature affect these relationships is important for developing new approaches to disease prevention and treatment. Understanding the propensity of certain bacterial species to grow at specific incubation temperatures also provides clues to a more detailed understanding of the causes and mechanisms of disease progression and to finding more effective treatments. Furthermore, contamination of foods via fecal contamination has been noted [19], and understanding the growth of gut bacteria that cause the food poisoning resulting from it could lead to improvements in food preparation and storage methods, thereby reducing the risk of foodborne illness.

In this study, we cultured the 51 most dominant gut commensal microbial species of the Japanese [20] and European [21] populations, which could be cultured simultaneously [22], and three harmful gut bacteria, *C. difficile*, *C. perfringens*, and *F. nucleatum* (Table 1), at 30–50°C and analyzed their growth.

MATERIALS AND METHODS

Bacterial strains

Bacteria were sourced from the American Type Culture Collection (ATCC), the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ), and the Japan Collection of Microorganisms (JCM; Table 1). The bacteria were cultured at 30°C, 33°C, 35°C, 37°C, 39°C, 41°C, 43°C, 45°C, and 50°C in an anaerobic chamber (10% CO_2, 10% H_2, 80% N_2; Invivo$_2$ 400, Ruskinn Technology, Bridgend, UK).

Culture systems

Microorganisms were cultured using the same methods as reported in previous studies [22]. Specifically, Gifu anaerobic medium (GAM; Nissui Pharmaceutical, Tokyo, Japan) was autoclaved at 115°C for 15 min and then promptly transferred to a sealed container with AnaeroPack™-Anaero (Mitsubishi Gas Chemical, Tokyo, Japan) for overnight incubation to eliminate oxygen. For pre-culture, a GAM + blood medium (GB medium) was used that consisted of whole horse blood (whole horse blood debridement, Japan Bio Supply Center, Tokyo, Japan), which had been stored anaerobically in AnaeroPack™-Anaero. It was added to GAM at 5% (v/v) within an anaerobic chamber.

Bacterial strains from frozen glycerol stock were initially inoculated into 96-well plates or vials in 500 µL or 3 mL GB medium, respectively, and then cultured at 37°C for 24–96 hr. In the case of pre-culture conducted in vials, 500 µL pre-culture was transferred to a 96-well plate, and approximately 2 µL from each culture was inoculated into 500 µL GAM in a 96-well plate, using a copy plate stand (Token, Chiba, Japan).

After 48 hr of anaerobic culturing, proliferation was assessed by measuring the optical density at 600 nm (OD_{600}) using a Thermo Scientific™ Multiskan™ GO (Thermo Fisher Scientific, Waltham, MA, USA). The percentage effect on proliferation was calculated by dividing the OD_{600} of the bacteria cultured at each temperature by the OD_{600} of the bacteria cultured at 37°C and then multiplying the result by 100.

Statistical analysis

Statistical analyses were performed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). The analyses were performed using a test for differences in population proportions. The experiment was performed with n=3 for each temperature group, and statistical analyses were performed on the OD_{600} at each culture temperature versus the OD_{600} at 37°C. For all data shown, ** and * indicate p-values, calculated by t-test, <0.01 and <0.05, respectively.

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Table 1. Occupancy rank and strain numbers used in this study

Occupancy rank		Bacterial species	Strain
European [21]	Japanese [20]		
1	10	*Bacteroides uniformis*	JCM 5828^T
3	25	*Parabacteroides merdae*	JCM 9497^T
4	12	*Dorea longicatena*	DSM 13814^T
5		*Ruminococcus bromii*	ATCC 27255^T
6		*Bacteroides caccae*	JCM 9498^T
8	49	*Bacteroides thetaiotaomicron*	JCM 5827^T
10	20, 26	*Ruminococcus torques*	ATCC 27756^T
13	23	*Faecalibacterium duncaniae*	JCM 31915
14	50	*Ruminococcus lactaris*	ATCC 29176^T
15	9	*Collinsella aerofaciens*	JCM 7790
16	18	*Dorea formicigenerans*	ATCC 27755^T
17	13	*Bacteroides vulgatus*	JCM 5826^T
18	37	*Roseburia intestinalis*	DSM 14610^T
20		*Eubacterium siraeum*	ATCC 29066^T
21	16	*Parabacteroides distasonis*	JCM 5825^T
23	42	*Bacteroides ovatus*	JCM 5824^T
26	5	*Eubacterium rectale*	JCM 17463
27		*Bacteroides xylanisolvens*	JCM 15633^T
28	39	*Coprococcus comes*	ATCC 27758^T
31		*Eubacterium ventriosum*	ATCC 27560^T
32	22	*Phocaeicola dorei*	JCM 13471^T
33	19, 34	*Blautia obeum*	DSM 25238^T
34		*Subdoligranulum variabile*	DSM 15176^T
35		*Pseudoflavonifractor capillosus*	ATCC 29799^T
38		*Holdemania filiformis*	DSM 12042^T
39		*Bacteroides stercoris*	JCM 9496^T
42		*Bacteroides eggerthii*	JCM 12986^T
43		*Butyrivibrio crossotus*	DSM 2876^T
44		*Bacteroides finegoldii*	JCM 13345^T
45		*Parabacteroides johnsonii*	JCM 13406^T
47	28	*Clostridium nexile*	ATCC 27757^T
49		*Anaerotruncus colihominis*	JCM 15631^T
50	14	*Ruminococcus gnavus*	ATCC 29149^T
51		*Bacteroides intestinalis*	JCM 13265
52	33	*Bacteroides fragilis*	JCM 11019^T
53		*Clostridium asparagiforme*	DSM 15981^T
54		*Enterococcus faecalis*	ATCC 700802
55		*Clostridium scindens*	JCM 6567^T
56		*Blautia hansenii*	JCM 14655^T
	3	*Bifidobacterium longum*	JCM 1217^T
	4	*Bifidobacterium pseudocatenulatum*	JCM 1200^T
	7	*Bifidobacterium adolescentis*	JCM 1275^T
	11	*Anaerostipes hadrus*	DSM 3319^T
	24	*Flavonifractor plautii*	ATCC 29863^T
	27	*Roseburia inulinivorans*	DSM 16841^T
	30	*Streptococcus salivarius*	JCM 5707^T
	35	*Clostridium bolteae*	JCM 12243^T
	41	*Clostridium innocuum*	JCM 1292^T
	43	*Coprococcus catus*	ATCC 27761^T
	45	*Enterocloster clostridioformis*	JCM 1291^T
	46	*Roseburia hominis*	JCM 17582^T
		Clostridioides difficile	JCM 1296^T
		Clostridium perfringens	JCM 1290
		Fusobacterium nucleatum	JCM 8532^T

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RESULTS

Bacteria that showed changes in growth when cultured at 30°C

At 30°C, 10 (20%) and 16 (31%) dominant bacterial species of human gut microbiota showed increased and inhibited growth, respectively, compared with at 37°C. The growth of the remaining species (49%) was unaffected (Fig. 1A).

B. fragilis (by 14%), *Bacteroides intestinalis* (by 58%), *Bacteroides xylanisolvens* (by 8%), *Clostridium bolteae* (by 30%), *Clostridium innocuum* (by 27%), *Clostridium scindens* (by 38%), *Dorea longicatena* (by 24%), *Enterococcus faecalis* (by 31%), and *Parabacteroides distasonis* (by 10%) showed statistically significantly increased growth ($p<0.05$; Fig. 2). On the other hand, *Anaerostipes hadrus* (by 57%), *Bifidobacterium pseudocatenulatum* (by 55%), *Blautia hansenii* (by 29%), *Blautia obeum* (by 88%), *Coprococcus catus* (by 95%), *Dorea formicigenerans* (by 59%), *Eubacterium rectale* (by 43%), *Flavonifractor plautii* (by 32%), *Holdemania filiformis* (by 23%), *Parabacteroides merdae* (by 42%), *Pseudoflavonifractor capillosus* (by 66%), *Roseburia hominis* (by 100%), *Roseburia inulinivorans* (by 100%), *Ruminococcus bromii* (by 98%), *Streptococcus salivarius* (by 42%), and *Subdoligranulum variabile* (by 78%) showed statistically significantly inhibited growth ($p<0.05$; Fig. 2).

Bacteria that showed changes in growth when cultured at 33°C

At 33°C, 7 (14%) and 12 (24%) dominant bacterial species of human gut microbiota showed increased and inhibited growth, respectively, compared with at 37°C. The growth of the remaining species (65%) was unaffected (Fig. 1B).

B. intestinalis (by 41%), *C. innocuum* (by 27%), *C. scindens* (by 35%), *Collinsella aerofaciens* (by 30%), *E. faecalis* (by 10%), and *P. distasonis* (by 14%) showed statistically significantly increased growth ($p<0.05$; Fig. 2). On the other hand, *Bacteroides finegoldii* (by 11%), *Bacteroides thetaiotaomicron* (by 21%), *B. pseudocatenulatum* (by 28%), *B. hansenii* (by 36%), *B. obeum* (by 17%), *Butyrivibrio crossotus* (by 17%), *D. formicigenerans* (by 80%), *E. rectale* (by 45%), *R. hominis* (by 96%), *R. inulinivorans* (by 42%), *S. salivarius* (by 35%), and *S. variabile* (by 78%) showed statistically significantly inhibited growth ($p<0.05$; Fig. 2).

Bacteria that showed changes in growth when cultured at 35°C

At 35°C, 1 (2%) and 8 (16%) dominant bacterial species of human gut microbiota showed increased and inhibited growth, respectively, compared with at 37°C. The growth of the remaining species (82%) was unaffected (Fig. 1C).

R. hominis (by 54%) showed statistically significantly increased growth ($p<0.05$). However, *B. fragilis* (by 9%), *B. pseudocatenulatum* (by 32%), *B. crossotus* (by 24%), *Clostridium asparagiforme* (by 15%), *E. rectale* (by 33%), *Parabacteroides johnsonii* (by 7%), and *S. salivarius* (by 20%) showed statistically significantly inhibited growth ($p<0.05$; Fig. 2).

Bacteria that showed changes in growth when cultured at 39°C

At 39°C, 11 (22%) dominant bacterial species of human gut microbiota showed inhibited growth compared with at 37°C. The growth of the remaining species (78%) was unaffected (Fig. 1D).

Anaerotruncus colihominis (by 23%), *Bacteroides eggerthii* (by 28%), *B. intestinalis* (by 17%), *Bacteroides ovatus* (by 18%), *B. thetaiotaomicron* (by 26%), *Clostridium nexile* (by 26%), *C. scindens* (by 17%), *C. catus* (by 23%), *Coprococcus comes* (by 17%), *P. distasonis* (by 7%), *Phocaeicola dorei* (by 24%), and *R. hominis* (by 35%) showed statistically significantly inhibited growth ($p<0.05$; Fig. 2).

Bacteria that showed changes in growth when cultured at 41°C

At 41°C, 2 (4%) and 13 (25%) dominant bacterial species of human gut microbiota showed increased and inhibited growth, respectively, compared with at 37°C. The growth of the remaining species (71%) was unaffected (Fig. 1E).

B. pseudocatenulatum (by 11%) and *R. bromii* (by 1%) showed statistically significantly increased growth ($p<0.05$; Fig. 2). On the other hand, *A. colihominis* (by 49%), *B. intestinalis* (by 24%), *B. xylanisolvens* (by 19%), *Bifidobacterium adolescentis* (by 36%), *B. obeum* (by 15%), *C. comes* (by 12%), *P. distasonis* (by 16%), *P. dorei* (by 15%), *P. capillosus* (by 18%), *Ruminococcus gnavus* (by 18%), *Ruminococcus torques* (by 39%), *S. salivarius* (by 17%), and *S. variabile* (by 24%) showed statistically significantly inhibited growth ($p<0.05$; Fig. 2).

Bacteria that showed changes in growth when cultured at 43°C

At 43°C, 17 (33%) dominant bacterial species of human gut microbiota showed inhibited growth compared with at 37°C. The growth of the remaining species (67%) was unaffected (Fig. 1F).

A. colihominis (by 22%), *B. intestinalis* (by 31%), *B. xylanisolvens* (by 19%), *B. adolescentis* (by 93%), *B. pseudocatenulatum* (by 11%), *B. obeum* (by 15%), *Eubacterium siraeum* (by 32%), *F. plautii* (by 36%), *P. distasonis* (by 27%), *P. dorei* (by 15%), *P. capillosus* (by 18%), *R. inulinivorans* (by 97%), *R. bromii* (by 20%), *R. gnavus* (by 19%), *R. torques* (by 47%), *S. salivarius* (by 17%), and *S. variabile* (by 24%) showed statistically significantly inhibited growth ($p<0.05$; Fig. 2).

Bacteria that showed changes in growth when cultured at 45°C

At 45°C, 1 (2%) and 41 (80%) dominant species of human gut microbiota showed increased and inhibited growth, respectively, compared with at 37°C. The growth of the remaining species (18%) was unaffected (Fig. 1G).

C. scindens (by 38%) showed statistically significantly increased growth ($p<0.05$). However, *A. colihominis* (by 92%), *Bacteroides caccae* (by 88%), *B. eggerthii* (by 72%), *B. finegoldii* (by 44%), *B. fragilis* (by 66%), *B. intestinalis* (by 100%), *B. ovatus* (by 46%), *Bacteroides stercoris* (by 26%), *B. thetaiotaomicron* (by 94%), *Bacteroides uniformis* (by 100%), *Bacteroides vulgatus* (by 100%), *B. xylanisolvens* (by 100%), *B. adolescentis* (by 100%), *Bifidobacterium longum* (by 100%), *B. pseudocatenulatum* (by 56%), *B. obeum* (by 100%), *B. crossotus* (by 55%), *C. bolteae* (by 98%), *C. nexile* (by 100%), *C. aerofaciens* (by 100%), *D. formicigenerans* (by 90%), *D. longicatena* (by 100%), *Enterocloster clostridioformis* (by 51%), *E. faecalis* (by 53%), *E. rectale* (by 100%), *E. siraeum* (by 100%), *Eubacterium ventriosum* (by 49%), *Faecalibacterium duncaniae* (by 97%), *F. plautii* (by 84%), *H. filiformis* (by 19%), *P. distasonis* (by 99%), *P. johnsonii* (by 63%), *P. merdae* (by 98%), *P. dorei* (by 39%), *Roseburia intestinalis* (by 100%), *R. inulinivorans* (by 99%), *R. bromii* (by 99%), *R. torques* (by 95%), *S. salivarius* (by 100%), and *S. variabile* (by 45%) showed statistically significantly inhibited growth ($p<0.05$; Fig. 2).

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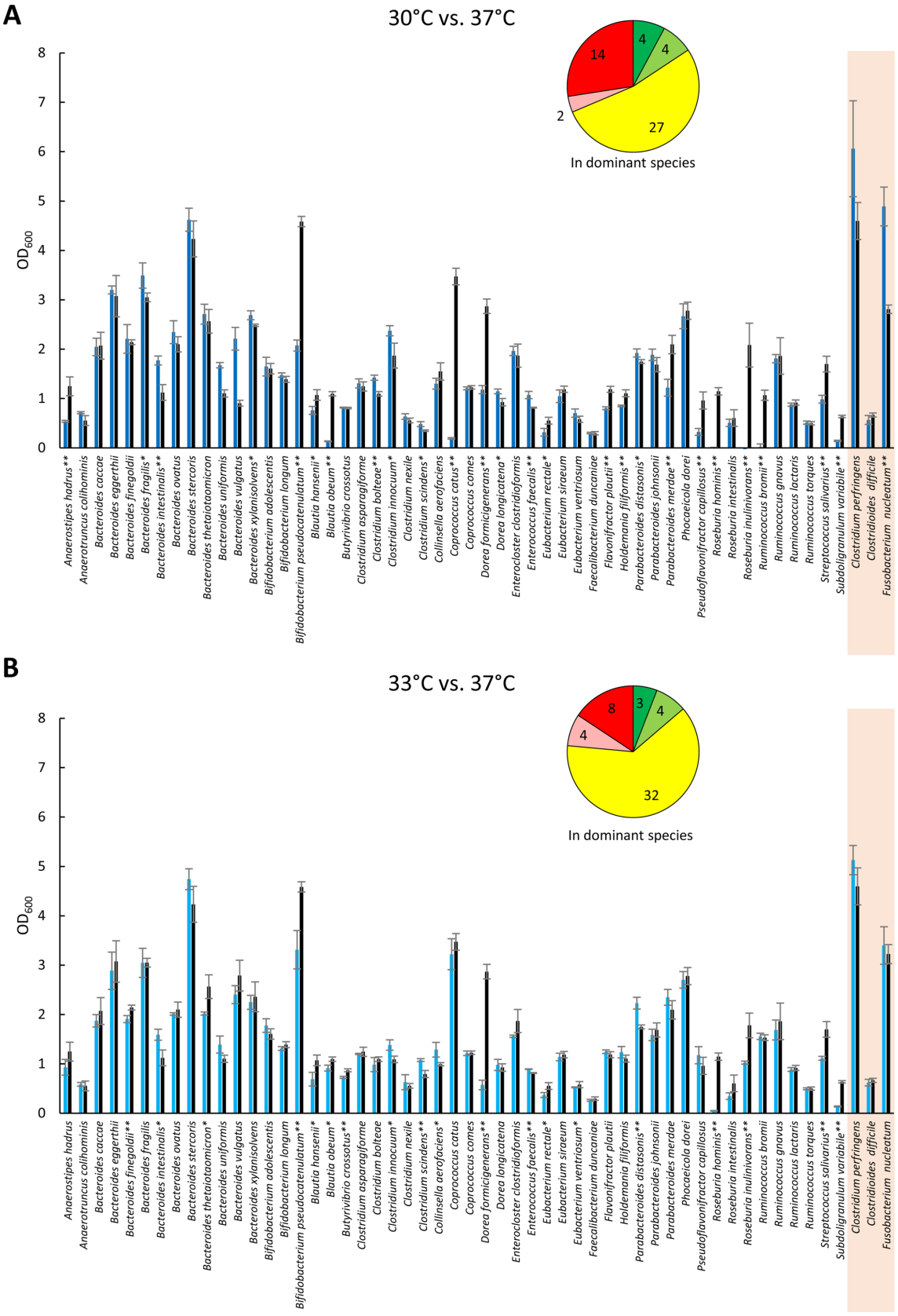

Fig. 1. Optical density at 600 nm (OD_{600}) values of different bacterial species cultured for 48 hr at (A) 30°C, (B) 33°C, (C) 35°C, (D) 39°C, (E) 41°C, (F) 43°C, (G) 45°C, and (H) 50°C are shown in blue, light blue, green, yellow, orange, red, vermilion, and purple respectively, and compared with the OD_{600} values when the species were cultured at 37°C (black). The pie chart compares the growth of the dominant bacterial species of human gut microbiota cultured at 37°C and those cultured at different temperatures, demonstrating the proportions of the numbers of bacterial species that exhibited a statistically significant difference in proliferation. Species with statistically significant enhanced growth are shown in green ($p<0.01$) or light green ($p<0.05$), species with no significant difference in growth are shown in yellow, and species with statistically significant growth inhibition are shown in red ($p<0.01$) or pink ($p<0.05$). The vertical bar graph for harmful bacteria is displayed on an orange background.

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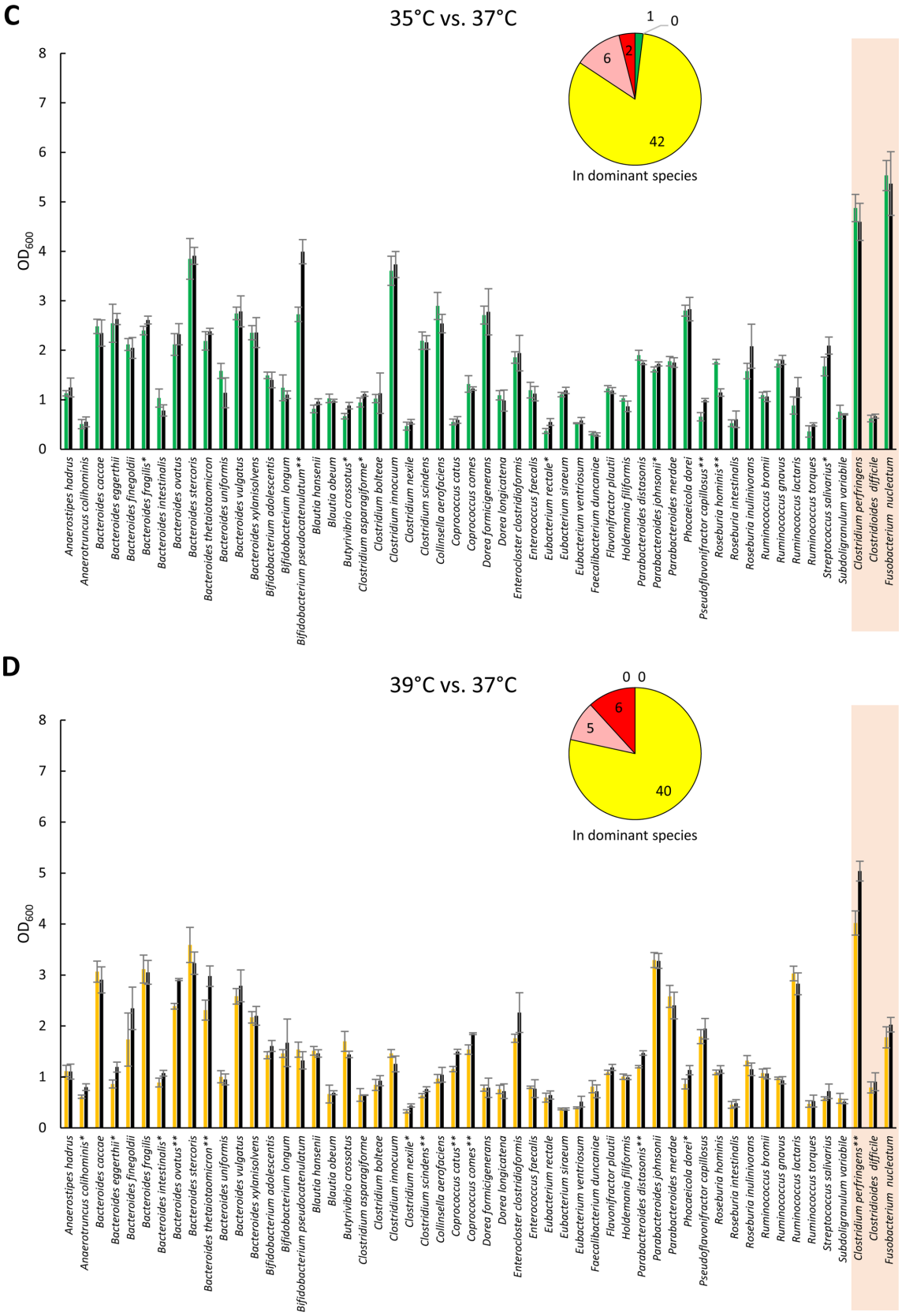

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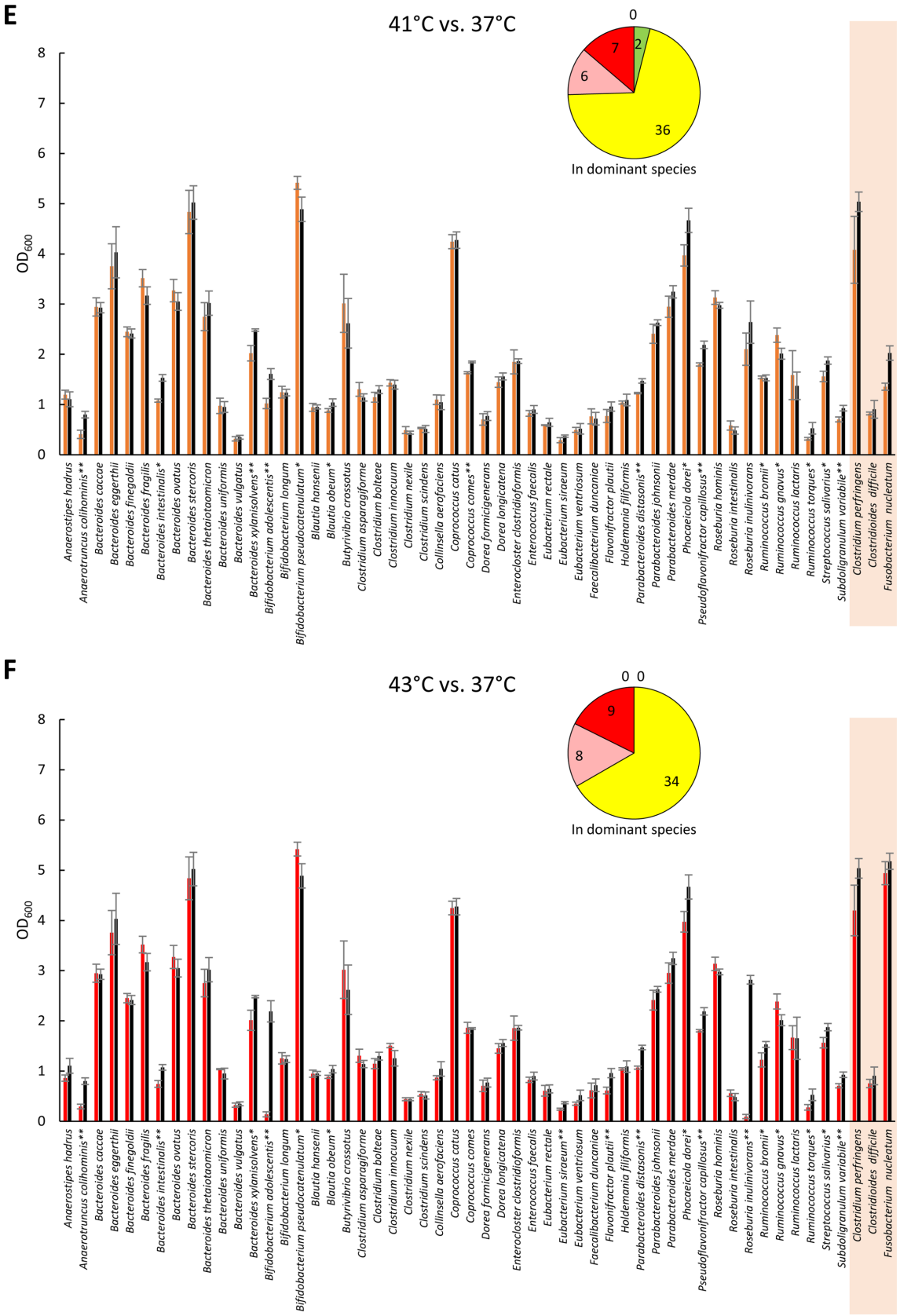

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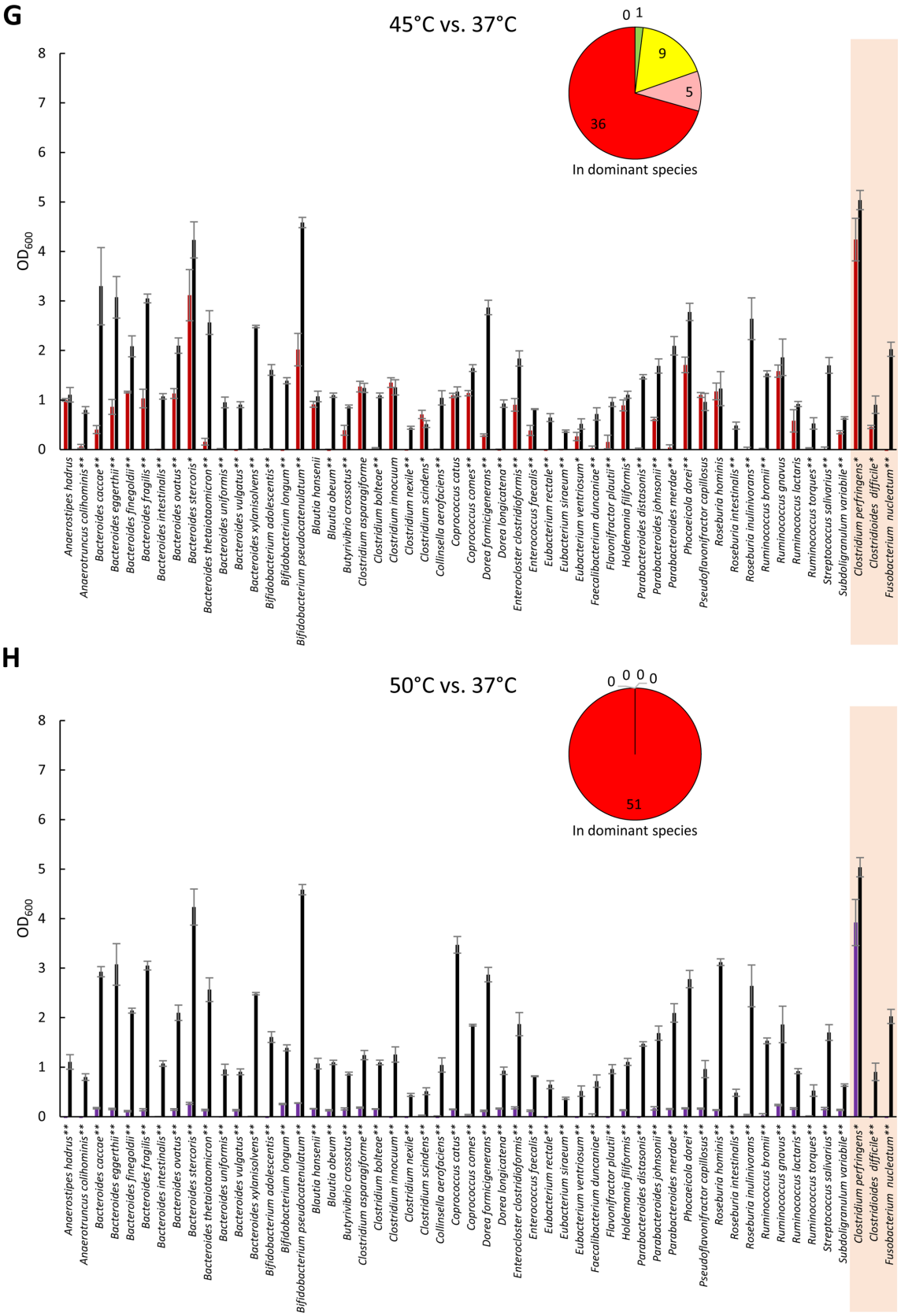

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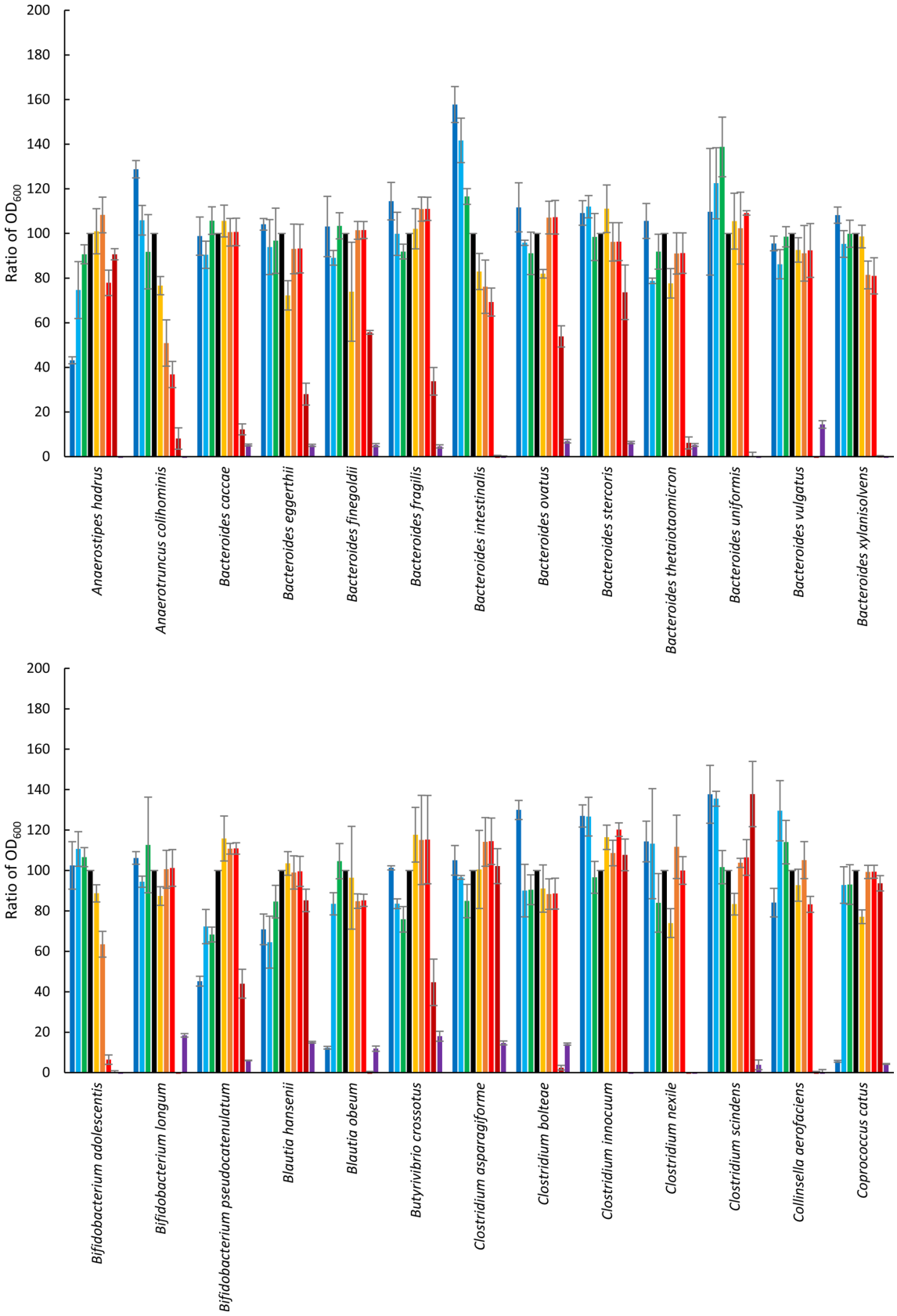

Fig. 2. Comparison of the optical density at 600 nm (OD_{600}) of each strain cultured at 37°C to those at the other temperatures. The effect of changing the culturing temperature from 37°C to each of the other temperatures on the growth of the most dominant species of human gut microbiota and harmful bacterial species was calculated by substituting the growth (OD_{600}) of the species at each culturing temperature and that at 37°C into the following formula: Effect on proliferation (%) = (OD_{600} of bacteria cultured at each temperature / OD_{600} of bacteria cultured at 37°C) × 100. Each bar in the graph represents the proliferation degree at various temperatures: 30°C (blue), 33°C (light blue), 35°C (green), 37°C (black, with 100 as the standard value in Fig. 2), 39°C (yellow), 41°C (orange), 43°C (red), 45°C (vermilion), and 50°C (purple). The vertical bar graph for harmful bacteria is displayed on an orange background.

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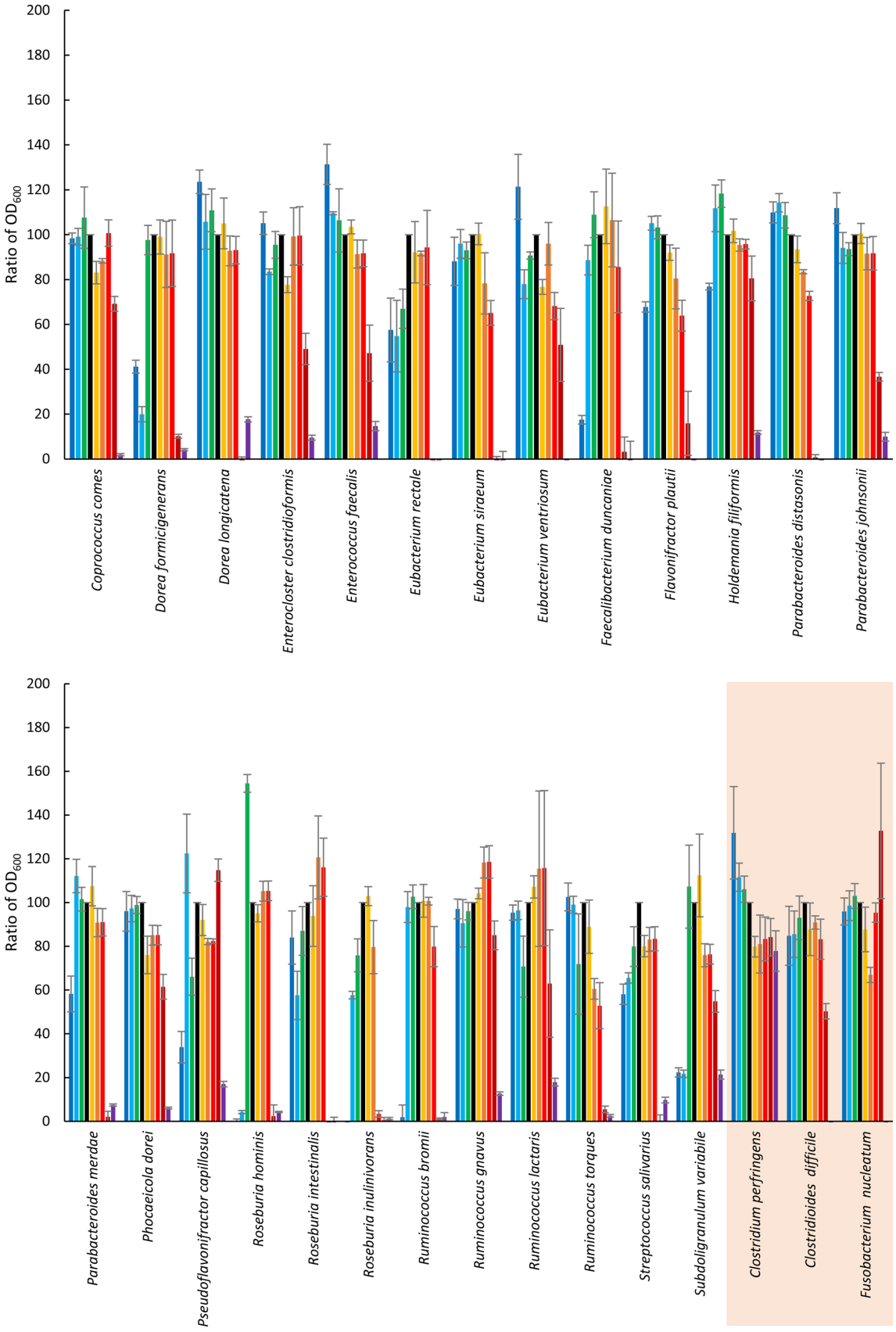

Fig. 2. Continued.

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Bacteria that showed changes in growth when cultured at 50°C

All 54 bacterial species showed statistically significantly inhibited growth. However, the growth inhibition percentage of *C. perfringen*s, a food poisoning bacterium, was lower than those of the other bacteria (22%; Figs. 1H, 2).

Temperatures at which the growth of the dominant species of the human gut microbiota changes substantially

A volcano plot was generated for each culture temperature with the p-value and fold change (OD_{600} at each temperature divided by OD_{600} at 37°C) when comparing the growth (OD_{600}) at each temperature versus that at 37°C on the vertical and horizontal axes, respectively. The threshold for determining that there was a substantial change in bacterial growth was set as a change in growth when $p<0.05$ plus at least a 30% decrease or increase (dashed lines in Fig. 3). There were substantial effects on the growth of 18 (35%), 8 (16%), 4 (8%), 3 (6%), 7 (14%), 41 (80%), and 51 (100%) dominant species of the human gut microbiota at 30°C (Fig. 3A), 33°C (Fig. 3B), 35°C (Fig. 3C), 41°C (Fig. 3E), 43°C (Fig. 3F), 45°C (Fig. 3G) and 50°C (Fig. 3H), respectively, but no substantial effects on the growth of any bacterial species at 39°C (Fig. 3D) based on the same criteria. Although growth was inhibited for most of the bacteria that showed effects on growth as a result of the changes in culture temperature, 4 (7.8%), 2 (3.9%), and 1 (2%) dominant species of the human gut microbiota showed substantially promoted growth at 30°C (Fig. 3A), 33°C (Fig. 3B) and 35°C (Fig. 3C), respectively.

DISCUSSION

In this study, gut bacteria were cultured in the range of 30°C to 50°C. However, the growth temperature for gut bacteria that can be in the host from the standpoint of the host viability temperature is 35°C to 41°C, and it is unlikely to be any other temperature in the living host body. Therefore, we will first discuss the bacterial species that showed promoted or inhibited growth within the host viability temperature range.

When cultured within the human viable body temperature range (35, 37, 39, and 41°C), almost all species did not show substantial fluctuations in growth (Fig. 3C–3E). This suggests that species adapt to survive within the human intestinal lumen or that those with growth temperatures corresponding to the human body temperature thrive as the predominant species in the human gut microbiota. Bacteria exhibiting growth that was substantially affected by changes in culturing temperatures within the range of human viable body temperatures (35, 37, 39, and 41°C) were selected using the thresholds mentioned earlier: change in growth if $p<0.05$ plus at least a 30% decrease or increase. Based on these thresholds, *A. colihominis*, *B. adolescentis, B. pseudocatenulatum*, *E. rectale*, *R. hominis*, and *R. torques* were selected as bacteria that showed inhibited growth. In contrast, no species exhibited substantial promotion of growth at these temperatures.

The growth of *E. rectale*, a known butyrate-producing bacterium, was statistically significantly inhibited (33% decrease, p=0.020; Fig. 3C) at 35°C. Butyrate induces regulatory T-cell differentiation and suppresses inflammatory bowel disease (IBD) [23]. *E. rectale* levels were also decreased in the intestines of patients with IBD [24]. These findings suggest that low body temperature may exacerbate IBD by reducing butyrate production associated with a reduced proportion of *E. rectale* in the gut microbiota. The growth of *B. pseudocatenulatum* was statistically significantly inhibited (33% decrease, p=0.0015; Fig. 3C) at 35°C. This bacterium does not produce butyrate [25]; however, in human feces, butyrate-producing bacteria increase with the increase in *Bifidobacterium* spp., and the butyrate concentration increases [26]. This suggests that the decrease in *B. pseudocatenulatum* proliferation with decreasing body temperature may be accompanied by a decrease in butyrate-producing bacteria, thus decreasing the butyrate concentration in the intestinal tract. The growth of *R. hominis*, a butyrate-producing bacteria [25], was statistically significantly inhibited (35% decrease, p=0.0037; Fig. 3D) at 39°C. *R. hominis* proliferation decreases after Coronavirus Disease 2019 (COVID-19) in the gut microbiota of patients with post-acute COVID-19 syndrome (long COVID) [27]. Moreover, the relative abundance of butyrate-producing bacteria decreased immediately after COVID-19 in patients with long COVID [28]. Taken together, the decrease in *R. hominis* associated with COVID-19-associated fever may cause long COVID. *A. colihominis*, the growth of which was statistically significantly inhibited (49% decrease, p=0.0031; Fig. 3F) at 41°C, possesses an enzyme that produces cysteine persulfide (CysSSH) from cystine. CysSSH is a reactive sulfur species (RSS), which is a highly antioxidant molecule, and enhances the antioxidant capacity of the host [29]. In addition, *A. colihominis* is a butyrate-producing bacterium [30, 31]. Collectively, the decrease in butyrate and antioxidant levels associated with the decrease in *A. colihominis* during fever may exacerbate intestinal inflammation. *R. torques*, the growth of which was statistically significantly inhibited (39% decrease, p=0.039; Fig. 3F) at 41°C, is a mucin-degrading bacterium and increases in patients with IBD [32]. It is possible that an increase in body temperature suppresses *R. torques* growth and improves IBD. In addition, *B. adolescentis*, the growth of which was statistically significantly inhibited (49% decrease, p=0.0024; Fig. 3F) at 41°C, is a species that is decreased in patients with Crohn's disease compared with those without the disease [24]. This suggests that *B. adolescentis* proliferation decreases with increasing body temperature, possibly causing Crohn's disease, a type of IBD.

The above summary suggests that hypothermia (35°C) exacerbates IBD by inhibiting the growth of *E. rectale*, which suppresses IBD [24], and *B. pseudocatenulatum*, which promotes the growth of butyrate-producing bacteria involved in IBD amelioration [26]. Hyperthermia (39 or 41°C) also inhibited the growth of butyrate-producing bacteria, *A. colihominis*, *R. hominis*, and *B. adolescentis*, the proliferation of which decreases in patients with Crohn's disease. This suggests that IBD could be exacerbated by hyperthermia as well as hypothermia, although with the exception that *R. torques*, which increases with IBD, is suppressed at 41°C [24].

The temperature ranges within which the host is not viable affected the growth of many gut bacteria. However, the growth of 27 (50%) and 36 (67%) species tested, including harmful bacteria, did not change statistically significantly at 30°C (Fig. 1A) and 33°C (Fig. 1B), respectively, low temperatures at which humans cannot survive. Moreover, the growth of 37 (69%) and 9 (18%) species did not change statistically significantly at 43°C (Fig. 1F) and 45°C (Fig. 1G), respectively, temperatures at which humans cannot survive. These results suggest that many dominant species of the gut commensal microbiota can grow outside the human body, regardless of temperature as long as they

are under anaerobic conditions. However, several species showed inhibited growth when cultured at specifically 30 or 33°C, which are below the body temperature at which humans can survive (Fig. 3A, 3B). The percentage of species that showed inhibited growth also increased when cultured at 43, 45, and 50°C, which are above the body temperature at which humans can survive (Fig. 3F–3H). Specifically, 17 (31%) species tested showed significantly inhibited growth when cultured at 43°C (Fig. 3F), while 42 (78%) and 53 (98%, excluding *C. perfringens*) species showed significantly inhibited growth when cultured at 45 and 50°C, respectively (Fig. 3G, 3H).

In summary, the temperature conducive to human viability is consistent with the optimal growth temperature of many gut bacteria. Conversely, certain species experience growth

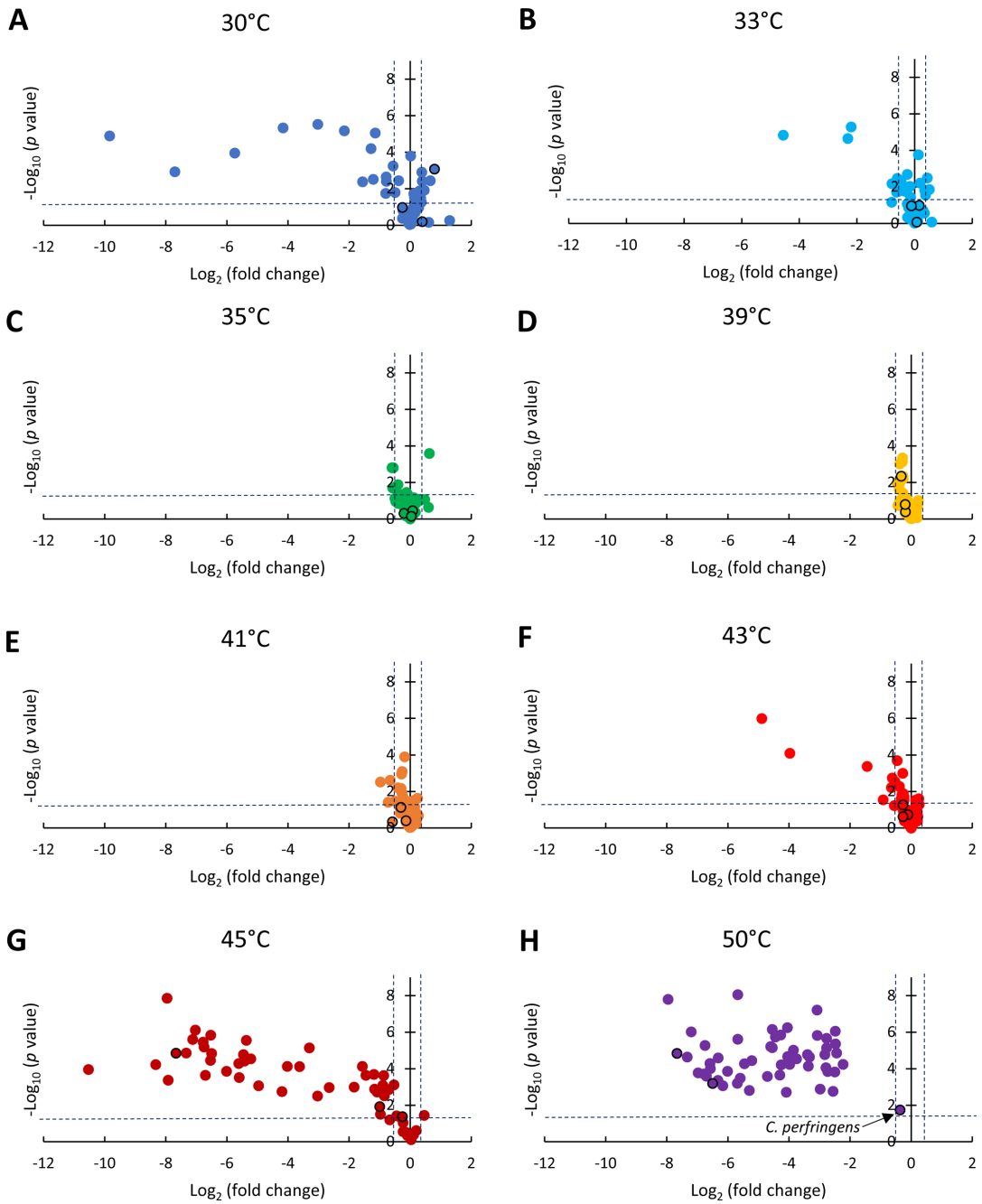

Fig. 3. The fold change of growth was obtained by dividing the growth of each of the 54 bacterial species at (A) 30°C, (B) 33°C, (C) 35°C, (D) 39°C, (E) 41°C, (F) 43°C, (G) 45°C, and (H) 50°C (n=3 for each temperature group) by the growth of the same bacteria at 37°C and was then presented on the horizontal axis as Log_2 (fold change). The p-value of the growth of each of the 54 bacterial strains cultured at (A) 30°C, (B) 33°C, (C) 35°C, (D) 39°C, (E) 41°C, (F) 43°C, (G) 45°C, and (H) 50°C (n=3 for each temperature group) against the growth of the same bacteria at 37°C is shown on the vertical axis as $-Log_{10}$ (p-value). Each species is depicted as a data point on the volcano plot. The horizontal dashed line shows $-Log_{10}$ (p-value) = 1.3 (p=0.05), and the vertical dashed lines show Log_2 (fold change) = −0.51 and 0.38 (± 30%). The dots showing data on harmful bacteria are bordered in black. For species that did not grow as a result of cultivation at each temperature, the OD_{600} was assumed to be 0.01, and Log_2 (fold change) was determined.

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suppression even under conditions viable for human growth, suggesting potential shifts in their relative abundances in response to body temperature fluctuations, thereby influencing the composition of the gut microbiota. Furthermore, the species that exhibit fluctuations in proliferation associated with body temperature fluctuations have been associated with disease, suggesting a potential link between these temperature-induced fluctuations and health outcomes.

The above results were obtained in this study by assessing the growth of each bacterium using the OD_{600} as an indicator; however, the OD_{600} has the problem that it does not reflect the growth of viable bacteria. Furthermore, the tested bacteria grow in competition with other bacteria in the intestinal tract *in vivo*, and the difference in growth due to changes in environmental temperature would be pronounced. On the other hand, there may be significant differences in bacterial behavior between the pure cultures in this study and the *in vivo* environment. Therefore, culturing gut bacteria at different culturing temperatures using fecal-injected human colonic organoids or fecal cultures, in which host cell immunity and competition with other bacteria would be reproduced, would provide insights in the future more similar to those obtained in the *in vivo* environment. Quantification of metabolites in culture supernatants of bacterial species that showed increased or inhibited growth with the change in culturing temperature in the future will be helpful in understanding the gut bacteria-mediated effects on host health associated with changes in body temperature.

Although gut bacteria are exposed to a wide range of temperatures as they exit the human gut into the environment, the growth of some gut bacteria was not significantly affected even at extreme incubation temperatures. *C. perfringens* causes food poisoning [33, 34]. While the growth of all bacterial species other than *C. perfringens* at 50°C was inhibited to less than 21% of that at 37°C, the growth of *C. perfringens* was maintained at 78% of that at 37°C (Figs. 2H, 3H). This suggests that fecal contamination with *C. perfringens* at a relatively high temperature, around 50°C, may cause exclusive *C. perfringens* proliferation. This potential exclusive *C. perfringens* growth may contribute to the frequent occurrence of *C. perfringens*-mediated food poisoning.

As gene disruption is an important tool for determining the physiological functions of bacteria, gene disruption of commensal intestinal bacteria such as bifidobacteria and *Escherichia coli* has been performed to elucidate the physiological functions of intestinal bacteria in detail. In the step of eliminating the temperature-sensitive plasmid used for gene disruption, the incubation temperature is often elevated to 40°C or higher [35, 36]. The temperature range at which human gut bacteria can grow, as revealed in this study, will provide useful information for the development of gene disruption methods for these bacteria.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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