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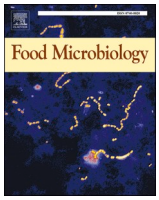

The effect of 3D printing speed and temperature on transferability of *Staphylococcus aureus* and *Escherichia coli* during 3D food printing

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ABSTRACT

The current study aimed to determine if the 3D-printing speed and temperature would impact the transferability of foodborne pathogens from the stainless-steel (SS) food cartridge to the 3D-printed food ink. *Staphylococcus aureus* and *Escherichia coli* were inoculated onto the interior surface of the SS food cartridges. Subsequently, a model food ink was extruded with a recommended macronutrient contribution of 55.8, 23.7, and 20.5% of carbohydrates, proteins, and fat, respectively. The impact of 3D-printing temperatures and speeds on transfer rates was analysed using a Two-Way ANOVA. *S. aureus* was transferred more from the cartridge to the food ink with a population of 3.39, 2.98, and 3.09 log CFU/g compared to 2.03, 2.06, and 2.00 log CFU/g for *E. coli* at 2000, 3000, and 4000 mm/s printing speed, respectively, at 25 °C. A Kruskal-Wallis Test was employed to investigate the effect of different speeds and temperatures on the transferability of *S. aureus* and *E. coli*. Speed was the main factor affecting *S. aureus* transferability, while temperature (25 and 50 °C) had the greatest impact on *E. coli* transferability. This research seeks to advance the understanding of 3D-printing parameters in pathogen transferability and help the food industry move towards this technology's quick and safe adoption.

1. Introduction

Investigation of the distribution of foodborne pathogens such as *Staphylococcus aureus* and *Escherichia coli* during the 3D printing process of food is essential for in-plant critical control points and food safety risk assessment as it is well-known that they are regularly implicated in foodborne outbreaks (Ekonomou et al., 2023). *S. aureus* is a gram-positive coccus and is one of the most important pathogens in humans and animals and a leading cause of foodborne disease worldwide (Ercoli et al., 2017; Ghodke et al., 2023). Control strategies are focused on hygiene measures to avoid food contamination and limit *S. aureus* growth (Fetsch and Johler, 2018). *E. coli* is a gram-negative, non-spore-forming rod and comprises a large and diverse group of bacteria (Jiang et al., 2024). *S. aureus* and *E. coli* can survive long periods in the environment and proliferate in most food products (Bintsis, 2017). Their transmission occurs when food or water that is contaminated is consumed. Outbreak investigations for *S. aureus* and *E. coli* remain challenging and would strongly benefit from additional data on their transferability when using novel food processing techniques, such as 3D printing. Previous research on the contamination of 3D food printers and 3D printed food by foodborne pathogens is currently very limited, showing the need for further studies to improve the safety of 3D printed food through microbial analysis.

In recent years, advances in research led to the broader application of the 3D printing process towards the production of personalised food. 3D printing is an additive manufacturing (AM) technique for fabricating a wide range of solid or semi-solid structures and complex shapes layer by layer (Ekonomou et al., 2024). To this end, 3D food printing technology allows the customisation and design of personalised food that can be used by individuals or specific population groups, such as athletes (Escalante-Aburto et al., 2021) and older adults (Pant et al., 2021; Ekonomou et al., 2024). Nowadays, 3D-printed food can be made from cereals (Vieira et al., 2020), fruits and vegetables (Ricci et al., 2019; Wedamulla et al., 2023), dairy products (Lanaro et al., 2017; Rianti-ningtyas et al., 2021) and meat and fish products (Cao et al., 2022; Dick et al., 2021). To achieve a good printability of the final product, the most crucial parameter is transforming any material or their combination into a formulation commonly called "food ink". The food ink should demonstrate viscoelastic behaviour with appropriate flow ability, allowing smooth extrusion and sufficient stability to retain its form after deposition (Ekonomou et al., 2024).

There is a growing interest in 3D-printed food due to its potential to

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simplify the supply chain, lower storage costs, and utilise all food materials (da Silva Costa et al., 2020; Tejada-Ortigoza and Cuan-Urquizo, 2022) while also promoting sustainability by valorising waste products (Baiano, 2022). The 3D printing technology in the food industry has been experiencing remarkable annual growth, and it is now approaching a global market worth nearly one billion dollars (Galanakis, 2024). Due to their specific characteristics and simplicity, 3D food printers have the potential to become a common household appliance, similar to the widespread acquisition of microwaves in the 1970s (McKevitt, 2019). However, this widespread will be synonymous to having multiple small-scale food processing or production facilities in private residences. This would significantly impact the food control system's resilience and adaptability, so policymakers must take proactive measures to assess and develop monitoring and control measures to limit the emergence of new pathogens and the re-emergence of recognised hazards in novel food vehicles or matrices. This is crucial to ensure the safety of the food supply chain and protect public health (McKevitt, 2019).

The available literature on the transferability of foodborne pathogens during the 3D printing of food is limited (Hamilton and Gibson, 2023a, 2023b, 2023c). To the best of our knowledge, this is the first study investigating the transferability of *S. aureus* and *E. coli* during the 3D printing of food. The detection of foodborne pathogens in the food industry is crucial in day-to-day practice to ensure safe food. To ensure the safety of 3D-printed food, international organisations and national government administrations must act proactively and focus on the collection of data and risk analyses to propose strategies such as hazard analysis critical control point (HACCP) systems in the food industry, which can significantly improve the level of food safety (Fu et al., 2021).

In the emerging field of 3D-printed foods, understanding and mitigating the microbiological risks involved in the application of this technology is vital. Herein, the current study aimed to evaluate the effect of various 3D printing speeds (2000, 3000, and 4000 mm/s) and temperatures (9, 25, and 50 °C) on the transfer rates of *S. aureus* and *E. coli* during 3D printing from the inoculated stainless-steel (SS) cartridge to a food ink model composed of a balanced macronutrient composition of 55.8, 23.7, and 20.5% of carbohydrates, proteins, and fat, respectively.

2. Materials and methods

2.1. Raw materials and food ink preparation

Commercially available whole-wheat bread, fresh banana, honey, and extra virgin Greek olive oil were purchased from a local retailer. The final food mixture was created by adding pure whey protein powder (Bulk Boost) and will now be referred to as food ink. The pure whey protein powder was purchased from Amazon, UK. All the nutritional facts for the ingredients used are shown in Table 1.

Based on established nutritional guidelines, a balanced diet should consist of 45–65% carbohydrates, 20–35% fats, and 10–35% protein. Keeping this in mind, a nutritionally balanced food ink was used, as a model, to investigate the impact of 3D printing parameters on pathogen transferability. A total of 97.5 g of whole-wheat bread, 90 g of fresh banana, 15 g of honey, 22.5 mL of Greek extra virgin olive oil, 30 g of pure whey protein powder, and 65 mL of distilled water were added in a cup and blended for 2 min to make a fine puree with no lumps using a Ninja Blender (Ninja Blender with Auto-iQ, 1000-Watt, Amazon, UK). The food ink was then transferred into sterile glass beakers, separated into portions of 30 g, and wrapped tightly to avoid any moisture evaporation before 3D printing.

Table 1
Nutritional facts per 100g of whole-wheat bread, banana, honey, extra virgin Greek olive oil, and pure whey protein powder.

	Whole-wheat bread	Extra virgin Greek olive oil	Pure whey protein powder	Banana	Honey
Energy (kcal)	278	824	387	89	307
Fat of which	6.6	91.6	7.0	0.3	0.0
Saturates (g)	0.6	14.0	2.5	0.1	0.0
Carbohydrate of which Sugars (g)	42.0 3.8	0.0 0.0	4.0 4.0	22.9 12.2	76.4 76.4
Fibre (g)	5.3	0.0	0.0	2.6	0.0
Protein (g)	10.0	0.0	77.0	1.1	0.4
Salt (g)	0.88	0.0	0.38	0.0	0.0

2.2. 3D-printed food process and parameters

A 3D food printer (Foodini, Natural Machines, Spain) with control-heated (0–90 °C) SS cartridges was used. The 3D printer consists of a fully closed print chamber and a maximum printing volume of 110 mm in height and 257 mm in diameter (Fig. 1). The samples were kept and printed at 9, 25, and 50 °C directly on a sterile Petri dish. The food ink was fed into the cartridge and pushed automatically through a nozzle with a diameter of 1.5 mm at scanning speeds of 2000, 3000, and 4000 mm/s. To evaluate the printability of each ink formulation, a shape comprised of numerous dots ranging from 0.5 to 2.0 g each was created. The reason for this shape was to allow getting consistent printing results using the different printing parameters.

2.3. Bacterial cultivation

S. aureus strain NCTC 12981 and *E. coli* NCTC Tox-12900 were activated separately from a frozen stock maintained at −80 °C in Cryoinstant vials with porous beads (Microbank, Pro-Lab Diagnostics, UK). A single bead for each strain was transferred separately to 10 mL brain heart infusion broth (BHI, Oxoid, UK, CM1135) and incubated overnight at 37 °C. From the overnight cultures, a volume of 0.1 mL for each strain was transferred separately in 10 mL BHI and incubated at 37 °C for 24 h. To prepare the working cultures, the cells were harvested by centrifugation at 6500×*g* for 10 min, washed twice with phosphate-buffered saline (pH 7.4; PBS), and finally resuspended in 10 mL of PBS to a final population of 10^9 Log CFU/mL before use.

2.4. Inoculation of the food ink cartridges

Initially, the cartridges were placed in sterilisation pouches 130 × 250 mm (Steris, UK) and autoclaved for 15 min at 121 °C prior to inoculation. Then, an inoculum of 0.1 mL of *S. aureus* strain NCTC 12981 or E. coli NCTC Tox-12900 containing 9.0 Log CFU/mL was added to the surface of the SS cartridge. The inoculum was evenly spread over the cartridge's interior surface with a sterile inoculation loop and allowed to dry completely in a biosafety cabinet for 30 min. Then, 30 g of the food ink was added to the cartridge, and the cartridge was placed back into the 3D printer and allowed to reach the appropriate temperature before 3D printing. All experiments were performed twice with three technical replicates. All results were expressed as Log CFU/g.

2.5. Bacterial enumeration and growth conditions

After the 3D printing process, ten (10) grams of sample were transferred aseptically from the Petri dish into a stomacher bag with 90 mL of Maximum Recovery Diluent (0.1% w/v peptone, 0.85% w/v NaCl; MRD) and homogenised at 200 rpm for 2 min using a Stomacher (400 Circulator Lab Blender, Seward, UK). Then, using the Whitley Automatic Spiral plater ('WASP 2'; Don Whitely Scientific Limited, Shipley, UK), an inoculum of 100 μl (set in logarithmic mode) was plated onto the appropriate selective medium in triplicate for each pathogen. Mannitol salt agar (Oxoid, UK, CM0085) was used as a selective medium for the enumeration of *S. aureus* after 24 h of incubation at 37 °C. MacConkey agar No.3 (Oxoid, UK, CM0115) was used as another selective medium to enumerate *E. coli* after 24 h of incubation at 35°C. All results were

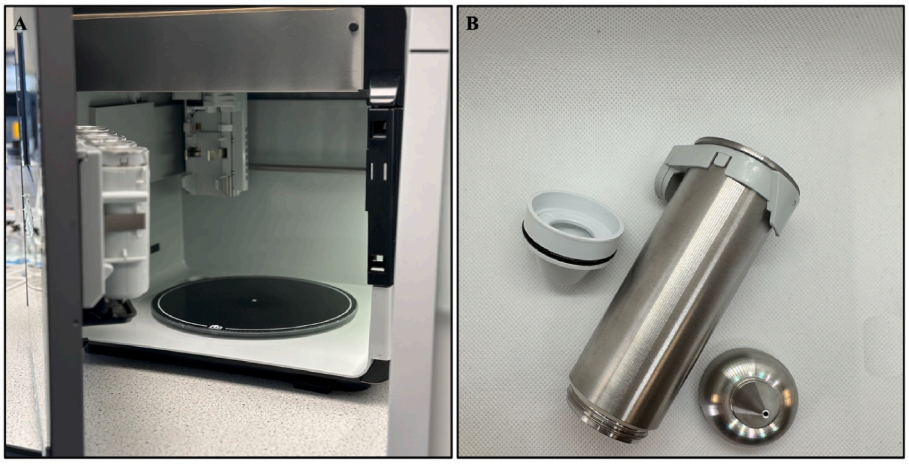

Fig. 1. Representative pictures of A) the Foodini 3D printer, which consists of a fully closed print chamber and the cartridge holder and B) the Foodini food ink cartridge with the top lid and the cartridge's nozzle of 1.5 mm.

expressed as Log CFU/g.

2.6. Sampling of the food ink cartridges

A wood cotton swab (Deltalab, Fisher Scientific, UK) was pre-moistened in PBS pH 7.4 and used to swab the cartridge's interior meticulously for 60 s after the 3D printing process under aseptic conditions. The swab was transferred to a 15 mL falcon tube with 3 mL of PBS and vortexed for 1 min. The appropriate ten-fold serial dilutions were performed in PBS.

For negative control, a cartridge was inoculated with 100 μL of sterile PBS. Similarly, for positive control, another cartridge was inoculated with 100 μL of either *S. aureus* or *E. coli*, separately. After inoculation, the samples were allowed to dry and then swabbed as described earlier to verify the population of the inoculated bacteria. The bacterial samples were then enumerated via plate counts at lower serial dilutions and from the spiral plate method.

2.7. Statistical analysis

All experiments were performed twice with three technical replicates. All data acquired were expressed as mean ± standard deviation (SD), and the Excel Microsoft® Office 365 (ver. 16.48) was used to plot graphs. Data were analysed using Minitab Statistical Software (Version 21.1.0) and the IBM® SPSS® statistics 26 software for macOS (SPSS Inc.).

A non-parametric rank sum test called Kruskal-Wallis (KW) was conducted to determine if there was a significant difference between the transferability of *S. aureus* and *E. coli* from the printer's cartridge to the food ink at different 3D printing temperatures and speeds. A two-way analysis of variance (ANOVA) was performed to determine the significance of temperature and speed as variables influencing the transfer rates of the pathogens. A KW test with post-hoc testing (Dunn's test with a Bonferroni Correction) and interaction plots were used to identify the interaction of the different speeds and temperatures on the transferability of the foodborne pathogens from the food cartridge to the food ink.

3. Results

3.1. Transfer of S. aureus and E. coli between the food cartridge and the food ink

The different printing temperatures affected the pathogens' transferability from the food cartridge to the food ink during the 3D printing process. Fig. 2 presents the transfer rates (Log CFU/g) of *S. aureus* and *E. coli* to the food ink during 3D printing at various speeds and temperatures.

At 2000 mm/s, the highest transferability of *S. aureus* was observed for 25 °C, reaching 3.39 Log CFU/g compared to 2.46 and 2.12 at 9 and 50 °C, respectively (Fig. 2, $p < 0.05$). The 3D printing speeds substantially affected the transferability of *S. aureus*, leading to a significantly lower population of 2.98 and 3.09 Log CFU/g when printed at 3000 and 4000 mm/s, compared to 3.39 Log CFU/g at 2000 mm/s, respectively (Fig. 2, $p < 0.05$). The opposite trend was observed for the lowest printing speed at 50 °C, where among all three temperatures, the population of *S. aureus* was consistently the lowest and slightly above (2.12 Log CFU/g) the detection limit of 2.0 Log CFU/g when printed at 2000 and 3000 mm/s, and below the detection limit at 4000 mm/s (Fig. 2, $p < 0.05$). The cross-contamination capacity appeared to differ considerably among the tested pathogens. In contrast to *S. aureus*, the highest transferability of *E. coli* was presented at 50 °C, reaching 2.83, 2.23, and 2.49 Log CFU/g when the food ink was printed at 2000, 3000, and 4000 mm/s, respectively (Fig. 2). The lowest transferability was observed at 9 and 25 °C with no significant differences between the two temperatures (Fig. 2, $p > 0.05$).

Among the tested 3D printing speeds and temperatures, at 3000 mm/s, a significantly lower population of *S. aureus* was recovered from the food cartridge at 9 °C when compared to 25 °C (4.23 and 4.53 Log CFU/g, respectively, Fig. 3, $p < 0.05$). At 4000 mm/s, the highest population of *S. aureus* was recovered after the 3D printing process at 25 °C (5.04 Log CFU/g). This was significantly higher than 9 and 50 °C at the same printing speed (Fig. 3, $p < 0.05$). On the other hand, the population of *E. coli* recovered from the food cartridge at 50 °C after printing at 3000 and 4000 mm/s was significantly lower (4.13 and 4.18 Log CFU/g, respectively) than at 2000 mm/s (Fig. 3, $p < 0.05$). The same trend was observed for *E. coli* counts recovered from the food cartridge at 25 °C. In all cases, no counts were detected for the negative controls. Our results reveal a clear effect on the transferability of different foodborne pathogens at diverse speeds and temperatures during the 3D printing process.

3.2. Transfer rates of S. aureus and E. coli during 3D printing

The KW test with post hoc tests and interaction plots were used to investigate the effect of different speeds and temperatures on the transferability of *S. aureus* and *E. coli* from the food cartridge to the food ink. The results are presented in Fig. 4A and B. Interaction plots show the relationship between one categorical variable and a continuous variable while taking the effects of a second categorical variable into account.

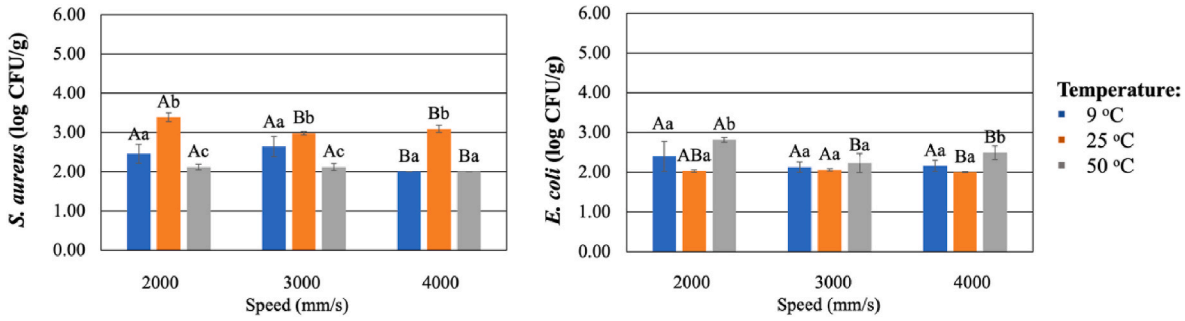

Fig. 2. Transfer (Log CFU/g) of *S. aureus* and *E. coli* from the food cartridge to the food ink after the 3D printing process at different speeds of 2000, 3000, and 4000 mm/s and temperatures at 9, 25, and 50 °C. Values represent the means, and the error bars represent the standard deviations (SD); n = 6. Different uppercase letters indicate significant differences between 3D printing speeds. Lowercase letters indicate significant differences between temperatures.

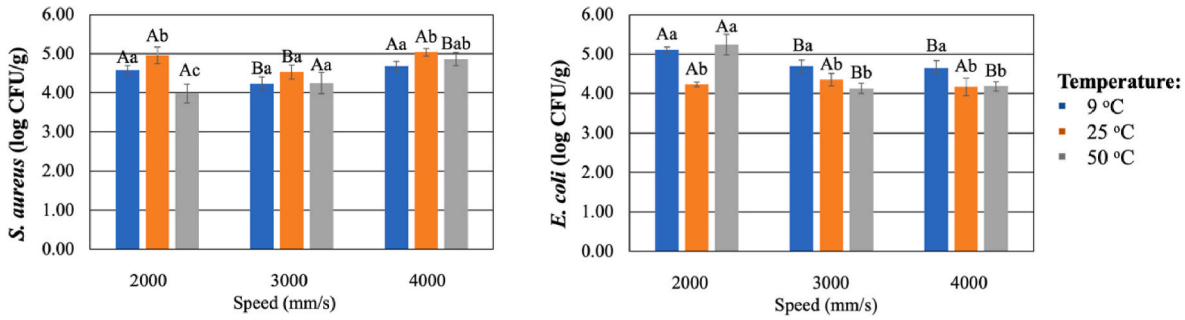

Fig. 3. Population (Log CFU/g) of *S. aureus* and *E. coli* recovered from the food cartridge after the 3D printing process of the model food ink at different speeds of 2000, 3000, and 4000 mm/s and temperatures at 9, 25, and 50 °C. Values represent the means, and the error bars represent the standard deviations (SD); n = 6. Different uppercase letters indicate significant differences between 3D printing speeds. Lowercase letters indicate significant differences between temperatures.

The feature of interactions is non-parallelism between the two lines. The different 3D printing temperatures used during the 3D printing process of the food ink showed the strongest interactions for *S. aureus*. More specifically, Fig. 4A (top right) shows that for temperatures 9 and 50 °C the speeds had similar effects on the transfer rates of *S. aureus*. The opposite effect was observed at 25 °C during printing at 3000 mm/s, where the speed was the main parameter that significantly affected the transferability of *S. aureus* (Fig. 4A, $p < 0.05$). In contrast, the plots in Fig. 4B display parallel lines without significant effect on the transferability of *E. coli* during 3D printing at 9 °C and all three speeds ($p > 0.05$). Despite this, the printing speeds of 2000 and 3000 mm/s at both 25 and 50 °C were found at a low level with a low inclination, revealing a significant effect on the transferability of *E. coli* (Fig. 4B, $p < 0.05$).

4. Discussion

3D food printing involves layer-by-layer deposition of edible materials to create three-dimensional structures (Ekonomou et al., 2024). While this innovative technology offers numerous advantages, including customisation and efficient resource utilisation, in industrial and commercial food settings, 3D printing technology introduces additional complexities. Microbial transfer may occur during the printing process and through contaminated raw ingredients, additives, or packaging materials. The interconnected nature of food production facilities demands a comprehensive understanding of the factors influencing pathogen transferability in the 3D printing context.

As 3D food printing technology continues to evolve, it is crucial to understand and address the potential food safety issues, such as the transferability of foodborne pathogens in home, commercial, and industrial environments. In our study, *S. aureus* and *E. coli* revealed the highest transferability from the food cartridge to the food ink during 3D printing at 25 and 50 °C, respectively, at the lowest speed of 2000 mm/s. Our results confirm that cross-contamination between food and surface is a multifactorial process strongly dependent on numerous factors, including the different bacterial species, surface material, and contact time (Gkana et al., 2017). Other studies have quantified the cross-contamination risk associated with the macronutrient composition of food inks in 3D food printing systems (Hamilton and Gibson, 2023c). Hamilton and Gibson (2023c) investigated the transfer kinetics of *Salmonella* Typhimurium, *Listeria monocytogenes*, and Tulane virus (TuV), from the food cartridge to the 3D-printed food ink with different macromolecular composition (e.g., butter, sugar, protein, and mixed composition). TuV was found to be transferred the most. Interestingly, no significant differences were observed between *L. monocytogenes* and *S.* Typhimurium regarding their transfer rates across different food matrices.

To the best of the authors' knowledge, our study is the first to investigate the transferability of foodborne pathogens during the 3D printing process at different speeds and temperatures. To this extent, KW test was applied to provide a scientific basis for the various interactions associated with the transferability of *S. aureus* and *E. coli* and the 3D printing parameters (speed and temperature) for risk management of such products. It was observed that temperature was the main parameter affecting the transfer rates of *S. aureus*, while the printing speeds of 2000 and 3000 mm/s at higher temperatures increased the transferability of *E. coli*. In general, the results of the present study showed that the population of *S. aureus* transferred from the cartridge to the food ink was approximately 1.0 Log CFU/g higher than *E. coli* at 25 °C during 3D printing at all speeds (2000, 3000, and 4000 mm/s). Midelet and Carpentier (2002) showed a stronger colonising capacity of gram-negative strains than the gram-positive strains on stainless steel food surfaces. The number of microorganisms that transfer to food from a contaminated surface depends on the density of microorganisms on the surface and their attachment strength on materials (Midelet and Carpentier, 2002). As follows, the higher attachment of *E. coli* compared to *S. aureus* cells to the food cartridge could explain the lower transferability during

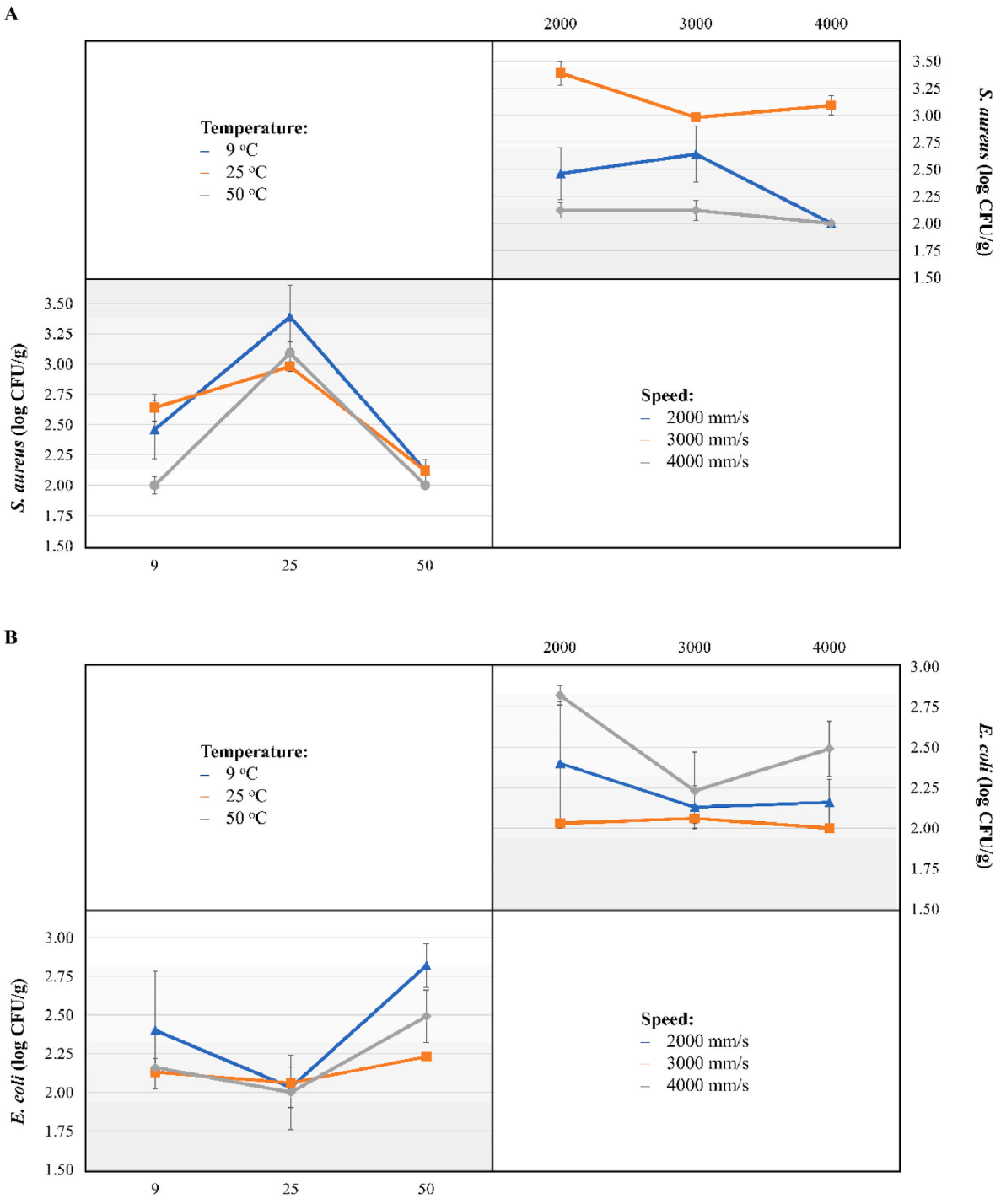

Fig. 4. Interaction plots determining the relationship between speeds (2000, 3000, and 4000 mm/s) and temperatures (9, 25, and 50 °C) and their impact on the transferability of A) *S. aureus* and B) *E. coli*.

the 3D printing process.

There is a limited number of studies investigating how 3D printing technology affects the transferability of foodborne pathogens. Recently, Hamilton and Gibson (2024) investigated the impact of storage conditions (20, 4, and −18 °C) on the inactivation of TuV during the extrusion of a protein cookie food ink. A significant reduction in virions was observed in capsules after 24 h at 4 and 20 °C. Even though this study was not directly linked to the transferability of pathogens or viruses during the 3D printing process, it provides significant insights into their recovery from the printer's cartridges and the effect of time and temperature. Numerous scientific studies have explored the transferability of pathogens to other non-contaminated food items like fresh-cut salads (Zilelidou et al., 2015), beef fillets (Gkana et al., 2017), salami (Sheen, 2008), and other deli meats (Sheen and Hwang, 2010) from infected surfaces, such as mechanical slicers and knives, and *vice versa*. The lack of scientific literature on the transferability of pathogens during the 3D printing process makes the comparison of our results challenging.

Existing technological bottlenecks and the lack of knowledge and harmonised norms (regulations, safety limits, etc., from the food safety authorities) regarding the hygienic features of the printers still hinder the use of 3D-printed food for mass production. In recent years, the European Union has made significant improvements in food legislation

to protect consumers throughout the food supply chain (Gkana et al., 2017). However, despite the updated Regulation (EU) 2015/2283 on novel foods that came into effect on January 1st, 2018, 3D-printed food products are not yet classified under this category (European Union, 2015). This is based on a summary of new (novel) food rules from 2018 that align with the principles mentioned above (European Union, 2021). According to the findings of the current study, *S. aureus* and *E. coli* were transferred from inoculated SS food cartridges to the non-inoculated food ink model through contact, and both speed and temperature had significant interactions. As a result, the authors suggest that a thorough investigation of cross-contamination phenomena should be carried out in future studies during the multifactorial process of 3D printing. This includes the transfer of various foodborne pathogens under different scenarios, such as the use of different bacterial species, surface and food materials, and multiple printing speeds and temperatures. The acquisition of quantitative data can play a crucial role in the prevention of pathogens' transferability during the cutting-edge 3D printing process of food. The data obtained in this study can support risk assessment and facilitate the development of effective risk management strategies for the food industry. As a result, the food chain can be safeguarded against the potential hazards that may arise from this novel food printing technology.

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CRediT authorship contribution statement

Sotirios I. Ekonomou: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sue Kageler:** Writing – review & editing, Validation, Formal analysis, Data curation. **Alexandros Ch Stratakos:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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