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A Girardeau, V Biscola, S Keravec, G Corrieu, F Fonseca. Application of Lactic Acid Bacteria in Time-Temperature Integrators. Lactic Acid Bacteria: A Functional Approach, Chapter 14, CRC Press, pp.241-260, 2020, 9780429422591. 10.1201/9780429422591 . hal-04027096

**HAL Id: hal-04027096**

**<https://hal.science/hal-04027096>**

Submitted on 13 Mar 2023

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# **Application of Lactic Acid Bacteria in Time-Temperature Integrators: *A Tool to Monitor Quality and Safety of Perishable Foods.***

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Published in “Albuquerque Cavalcanti de Albuquerque, M., de Moreno de LeBlanc, A., LeBlanc, J.G., & Bedani, R. (Eds.). (2020). *Lactic Acid Bacteria: A Functional Approach* (1st ed.). CRC Press. <https://doi.org/10.1201/9780429422591>  
Chapter 14, pp241-260.

## **Abstract**

Biological Time-Temperature Integrators (TTI) offer a novel approach for improving food safety and preventing spoilage. These smart tools relay, by an irreversible color shift, the cumulative effect of time and temperature on the microbial quality of food they are attached to. Among various types of TTI developed to date, biological TTI have the advantage of reproducing microbiological spoilage reactions that take place in food. They are based on the pH decline of a medium contained within the label, resulting from lactic acid bacteria (LAB) growth and acidification. When developing LAB-based TTI, careful LAB strain selection, research and development efforts on TTI production are necessary to closely match the behavior of both spoilage and pathogenic microorganisms that grow during the storage of perishable foods. Covering a wide range of time-temperature profiles is a challenging goal involving research in different domains (microbiology, food science, modelling, *etc.*). This chapter describes the design and working principle of LAB-based TTI, how they are parametrized to be able to track a wide range shelf-lives and how their performance is evaluated. Current applications and future prospects of this innovative way of using lactic acid bacteria are also discussed.

## **Introduction**

Fermented foods containing lactic acid bacteria (LAB) have been around for centuries. The ability of these microorganisms to acidify their environment by producing lactic acid gives them a strong preservative effect. In more recent years, this technological property of LAB is also being used as a tool to measure shelf-life of perishable foods.

Most refrigerated perishable foods are not sterile. The microorganisms they contain can multiply, degrade their environment and often produce unsavory metabolites. Their growth affects the microbiological quality of food and can lead to spoilage. Food quality thus evolves over time, more or less rapidly as a function of a number of parameters such as storage temperature, pH, water activity, gas composition, *etc.* Among these factors, temperature has the most critical effect on spoilage rates due to the rapid temperature changes that can take place during transport and storage. Ensuring cold chain integrity, from production to consumption

is therefore crucial to maintain and control the microbiological quality of refrigerated food. In practice, cold chain integrity is not always kept throughout production, distribution and domestic storage. Breaking the cold chain can affect expected shelf-life, making it difficult to predict the microbiological quality and safety of food at the time of consumption. Food industrials are responsible for the safety of products they have either imported, produced, processed, manufactured or distributed (EFSA Regulation No. 764/2008). Consequently, there is frequent conservative estimations of shelf-life by actors of the food industry who add substantial security margins that lead to considerable waste of perfectly good products. Among the 28 countries of the European Union, annual food waste attributed to date marking issues was estimated to be between 6.9 and 8.9 million tones, across the manufacturing, processing, retail and household sectors (European Commission, 2018). The emergence of innovative technologies has therefore been encouraged by the European Union to help reduce food waste, while still ensuring consumer safety.

Time-temperature integrators (TTI) can be considered as part of said innovative technologies. They are simple tools that provide information on the microbiological quality of a food product throughout the cold chain, based on its true time-temperature history. These smart devices are reliable shelf-life indicators that can contribute to reduce food waste, without compromising food safety. TTI can be used to track all kinds of products (food, medical and pharmaceutical products, cut flowers, *etc.*) for which storage and transportation at low temperatures are essential to ensure proper preservation (Kerry et al. 2006, Sahin et al. 2007, McMeekin et al. 2008). Despite having undergone significant technical evolutions with over 100 patents delivered since the 1960s, few prototypes were able to reach marketing stage (Sahin et al. 2007, Wang et al. 2015).

The present chapter describes the design and working principles of LAB-based time-temperature integrators then focuses on how they are configured to cover a wide range of shelf-lives. Current applications and future prospects of this innovative way of using lactic acid bacteria are also discussed.

## **Time-Temperature Integrators**

Time-temperature integrators are relatively small and cost-effective devices that relay by an irreversible and easily detectable sign, the cumulative effect of time and temperature on the food they are tracking. To be able to integrate all breaks of the cold chain during the food's entire life-cycle, and adjust shelf-life information accordingly, TTI generally come in the form of labels attached directly on to the food's packaging. These labels can be divided into three main categories, according to their working principles: chemical, physical or biological TTI (**table 1**). The first group comprises polymerization-based, photochromic-based and oxidation reaction-based systems (Galagan and Su 2008, Salman et al. 2009, Gou et al. 2010). Physical TTI encompass devices that are diffusion-based (Monitor Mark®, USA), nanoparticle-based, electronic, and so forth (Zweig 2005, Zeng et al. 2010). Biological TTI are based on enzymatic reactions and/or the use of microorganisms such as yeasts and lactic acid bacteria (Wang et al. 2015). Whatever its working principle is, a TTI's response is always strongly correlated to changes occurring within the tracked product's matrix, at a given time-temperature profile. Most of the commercialized TTI today are of the chemical or physical types (table 1), but biological TTI, and more specifically LAB-based TTI, are the ones that actually reproduce the microbiological reactions that take place in food, leading to spoilage.

**Table 1:** Examples of currently commercialized TTI belonging to different categories and their basic functioning principles.

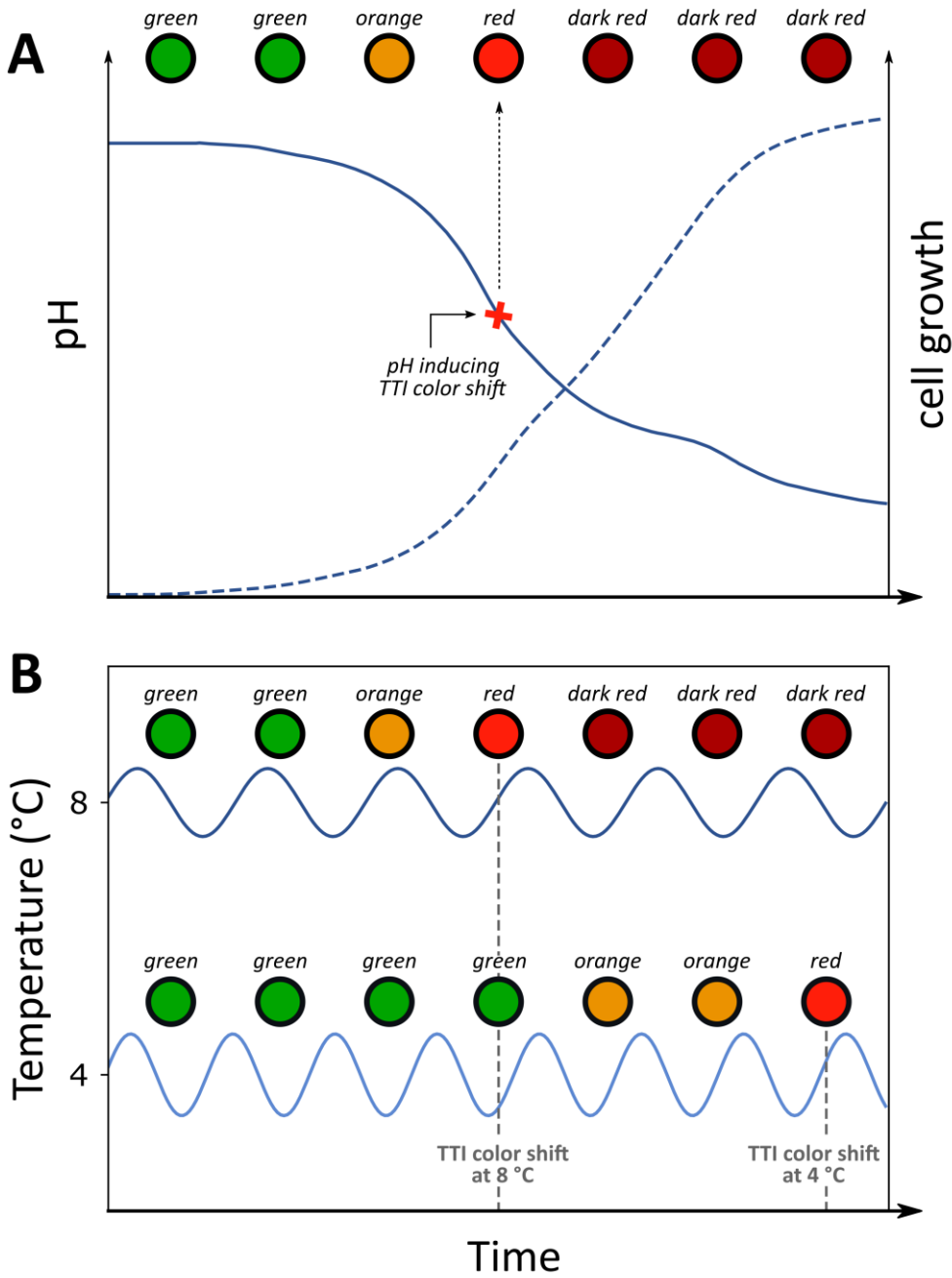
Commercial name / company	Category	Functioning principle
Fresh-Check® and HEATmarker™ (TempTime, New Jersey, USA)	Chemical	Polymerization-based
OnVu® (BASF, Ludwigshafen, DE)	Chemical	Photochromic-based
Freshpoint® (Freshpoint Quality Assurance, Neshar, IS)	Chemical	Photochromic-based
Monitor Mark® (3M, St. Paul, MN, USA)	Physical	Diffusion-based
Tempix® (TEMPIX, Gävle, SE)	Physical	Diffusion-based
Tsenso® (Tsenso, Stuttgart, DE)	Physical	Electronic
Koovea® (Koovea, Montpellier, FR)	Physical	Electronic
CheckPoint® (Vitsab, Limhamn, SE)	Biological	Enzymatic
TopCryo® (Cryolog, Nantes, FR)	Biological	LAB growth

### Design principle of lactic acid bacteria-based TTI

The active core of biological time-temperature integrators based on lactic acid bacteria contains three main components: a growth medium, lactic acid bacteria and a (or several) chromatic indicator(s). With these labels, alteration is generally determined by a progressive color shift brought about by a pH drop of the medium contained within the label, due to the growth and lactic acid production of LAB (**Fig. 1A**). Perishable food quality loss kinetics greatly depend on the growth and metabolic activity of the microorganisms they contain which in turn, are heavily influenced by temperature. The influence of temperature on the color shift kinetics of a biological TTI label is illustrated on figure 1B: the endpoint of a TTI label tracking a food product stored, for example, on the door of a household refrigerator (typically 8 °C) will be reached twice as fast as one tracking a similar food product placed at the back of the refrigerator (typically 4 °C).

Microorganisms contained within TTI labels must have similar growth and/or metabolic activity responses to temperature variations, as selected microorganisms contained within the food being tracked (e.g. spoilage LAB, *Listeria monocytogenes*, *Staphylococcus aureus*, etc). The underlying mechanisms of these labels therefore relies on predictive microbiology: the determination and validation of models for growth and metabolic activity of microorganisms as a function of various environmental conditions (temperature, pH, water activity, etc.). Examples of studies reported on the development of LAB-based TTI systems to monitor the quality and safety of perishable foods using different strains and varying designs, are presented in **table 2**. Although many examples of LAB-based TTI prototypes can be found in literature, only three have been taken to market to date: eO®, TRACEO® and TopCryo®. All were developed and

commercialized by Cryolog Clock-T° (Nantes, France). The present chapter will use Cryolog's latest self-adhesive label, TopCryo®, as a concrete example to explain LAB-based TTI parametrization.



**Figure 1:** Schematic representation of the growth (dotted line) and acidification activity (solid line) of LAB in a TopCryo® TTI label stored at 8 °C, inducing a color shift when a specific pH is reached (A) and the color shift's kinetic dependence on the average temperature profile: at 4 °C the color shift takes place later than at 8 °C (B). The color shift response is also dependent on the TTI's label composition.

**Table 2:** A few studies reported on the development of various LAB-based TTI systems to monitor the quality and safety of perishable foods using different LAB strains.

LAB used	Design of TTI system	Applicability and performance evaluation when available	References
<i>Lactobacillus sakei</i>	<ul style="list-style-type: none"> <li>- Laboratory screw-cap bottles containing nutrient rich growth medium, chlorophenol red and microorganisms;</li> <li>- Activation at inoculation of TTI medium;</li> <li>- Color shift from red to yellow, via orange.</li> </ul>	<ul style="list-style-type: none"> <li>- Modelling of TTI evolution isothermally between 0 °C and 16 °C;</li> <li>- Shelf-life studies correlating TTI endpoint with end of minced beef shelf-life.</li> </ul>	Vaikousi et al. 2008, 2009
<i>Carnobacterium maltaromaticum</i>	<ul style="list-style-type: none"> <li>- Thin, self-adhesive label containing a gel like growth medium, chromatic indicators and microorganisms;</li> <li>- Activation by rapid freeze-thaw at room temperature;</li> <li>- Color shift from green to red.</li> </ul>	<ul style="list-style-type: none"> <li>- Modelling of the growth and acidification activity of the strain used within the TTI label medium, as a function of temperature, assuring accurate TTI response time;</li> <li>- Shelf-life studies and challenge tests done on ground beef, minced chicken and smoked salmon ensuring that TTI endpoint corresponds to the tracked food's shelf-life.</li> </ul>	Ellouze et al. 2008, 2010, 2011 Louvet et al. 2005
<i>Weissella cibaria</i>	<ul style="list-style-type: none"> <li>- Laboratory cell culture-ware containing a nutrient rich growth medium, bromothymol purple, methyl red and microorganisms.</li> <li>- Activation at inoculation of TTI medium;</li> <li>- Color shift from purple to yellow.</li> </ul>	<ul style="list-style-type: none"> <li>- Strong correlation between Ea of the system's pH and color evolution and the quality degradation of ground beef and chicken breast.</li> </ul>	Kim et al. 2012 Park et al. 2013
<i>Weissella koreensis</i>	<ul style="list-style-type: none"> <li>- Laboratory test tubes containing MRS based growth medium, bromothymol blue, methyl red and microorganisms.</li> <li>- Color shift from green to red, via yellow.</li> </ul>	<ul style="list-style-type: none"> <li>- Strong correlation between Ea of TTI system and the Ea of Korean fermented food (kimchi) evolution;</li> <li>- TTI system thus proposed to indicate ripeness of kimchi.</li> </ul>	Lim et al. 2014
<i>Lactobacillus ssp.</i>	<ul style="list-style-type: none"> <li>- Two-dimensional code made of nanobeads containing microorganism immobilized on a nutrient rich base.</li> <li>- Time-temperature effect translated into both a color shift and an easily scannable 2D code.</li> <li>- Must be stored below 5 °C, gradually activated between 5 and 15 °C.</li> </ul>	<ul style="list-style-type: none"> <li>- Limited temperature range: 5 to 15 °C.</li> </ul>	Lee and Jung 2013
<i>Lactobacillus rhamnosus</i>	<ul style="list-style-type: none"> <li>- Transparent disk-shaped container including a nutrient rich growth medium, bromothymol green, methyl red and microorganisms.</li> </ul>	<ul style="list-style-type: none"> <li>- Proposed for commercial, chilled products.</li> </ul>	Lu et al. 2013

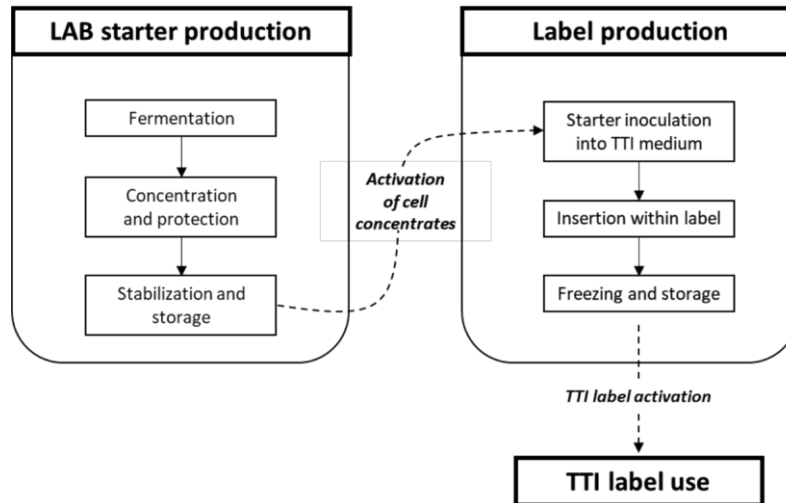
Ea: The Arrhenius activation energy parameter, quantifying a reaction's dependency to temperature.

TopCryo® was patented in 2005 (Louvet et al. 2005) as a device intended to monitor the quality of thermosensitive products. Their active core contains chromatic pH indicators and a gel-like growth medium inoculated with *Carnobacterium maltaromaticum* CNCM I-3298 cells, a psychrotrophic lactic acid bacteria. TopCryo® labels are meant to operate within a temperature range of 2 °C to 12 °C, whilst integrating breaks of the cold chain at room temperature. *C. maltaromaticum* cells contained within the label then start to multiply and acidify the growth medium. The acidification results in a color shift from bright green (pH > 8) to deep red (pH < 6.5), more or less rapidly, depending on the label’s time-temperature history (**Fig. 1**). TopCryo® labels are designed to cover different shelf-lives, ranging from 30 hours to 8 days (192 hr) at 4 °C (**table 3**), depending on the type of food that needs to be tracked. A given label (*e.g.* TopCryo® E) tracking perishable food products stored at a temperature below 4 °C, such as 2 °C, will reach its shelf-life endpoint slower than it would at 4 °C. Inversely, this same label will reach its shelf-life endpoint faster than at 4 °C if it is used for tracking foods stored at 12 °C.

**Table 3:** Shelf-life (in hours) of the TopCryo® TTI label range offered by Cryolog, as a function of storage temperatures. Information obtained from the Cryolog website (<http://cryolog.com/topcryo>), January 2019.

TopCryo® label range	Storage temperature				
	2°C	3°C	4°C	8°C	12°C
<b>TopCryo® B</b>	50	36	<b>30</b>	18	12
<b>TopCryo® C</b>	76	57	<b>48</b>	27	18
<b>TopCryo® D</b>	112	85	<b>72</b>	40	25
<b>TopCryo® E</b>	142	113	<b>96</b>	54	32
<b>TopCryo® F</b>	184	141	<b>120</b>	57	40
<b>TopCryo® G</b>	216	168	<b>144</b>	80	46
<b>TopCryo® H</b>	254	196	<b>168</b>	93	55
<b>TopCryo® I</b>	292	224	<b>192</b>	106	64

Producing a LAB-based TTI such as TopCryo® involves two main processes: the production of frozen LAB concentrates followed by the production of the TTI labels themselves (Fig. 2).



**Figure 2:** Diagram of LAB-based TTI production steps.

The production of frozen bacterial concentrates, such as *C. maltaromaticum*, includes the following key steps:

1. Fermentation: it is done in pure culture and batch mode. pH, temperature and harvest time are controlled and set to values that result in the growth of the highest bacterial population (biomass) exhibiting the expected acidification activity for a given TTI;
2. Concentration and protection: harvested cells are separated from the culture medium by centrifugation and resuspended in solutions containing protective molecules to reduce cell damage potentially caused by stabilization;
3. Stabilization and storage: the stabilization of bacterial concentrates by freezing or freeze-drying, enables the storage of LAB concentrates for long periods of time (6 to 24 months), without losing cell viability or any other technological function. Storage is generally done at -60°C to -80 °C for frozen concentrates or -20 °C to 4°C for freeze-dried concentrates.

The production of the LAB-based TTI label itself, includes the following key steps:

1. Starter inoculation into the TTI medium: in order to produce labels, the stored bacterial concentrates are freeze-thawed (or rehydrated) and a precise starter dosage is mixed into the label medium, depending on the desired shelf-life of the TTI being produced;
2. Insertion of medium within the label: this is achieved using a specially designed and patented machine that, in the case of TopCryo® TTI, seals the medium into self-adhesive labels (Vaillant and Peuteil, 2012)
3. Freezing and storage: the inoculated labels are then inactivated by blast freezing and stored at -40 °C until use. Activation is achieved by rapid freeze-thaw at room temperature upon placing the label on the food to be tracked.



## Parametrization

TTI label parametrization consists in identifying the production conditions to achieve a target shelf-life. It can be carried out according to various parameter modulation strategies involved in the TTI production process. So far, much of TTI shelf-life modulation – such as the range covered by TopCryo® labels – is achieved by adjusting the initial cell concentration inoculated into the label. There is indeed a negative linear relationship between initial cell concentration and the label's shelf-life response time. Topcryo®'s 8-day (192 hr) shelf-life at 4 °C (TopCryo® I) is reached by inoculating the lowest cell concentration allowing reproducible acidification rates. The 30-hour shelf-life is analogously achieved by inoculating the highest possible cell concentration into the label, beyond which the TTI's initial color would no longer be green. The color shift response times of 30 hours and 8 days at 4 °C correspond thus respectively to the shortest and the longest shelf-life that can be achieved by these labels. However, time-temperature integrators are ideally meant to track the microbiological quality of different food types displaying varying lengths of shelf-lives outside of the range offered by TopCryo®, such as 3 to 21 days for various meats and 0.5 to 3 days for shellfish (Dalgaard, 1995). To be used at a much larger scale, LAB-based TTI are thus expected to cover a wider shelf-life range than they currently do. Three different TTI parametrization strategies can be pursued to further increase response time ranges, beyond simply adjusting initial cell concentration: (1) modulating the physiological state of the selected strain; (2) tweaking the label medium's composition; (3) using a different bacterial strain or species alone or altogether.

### *Modulating the physiological state of the selected strain*

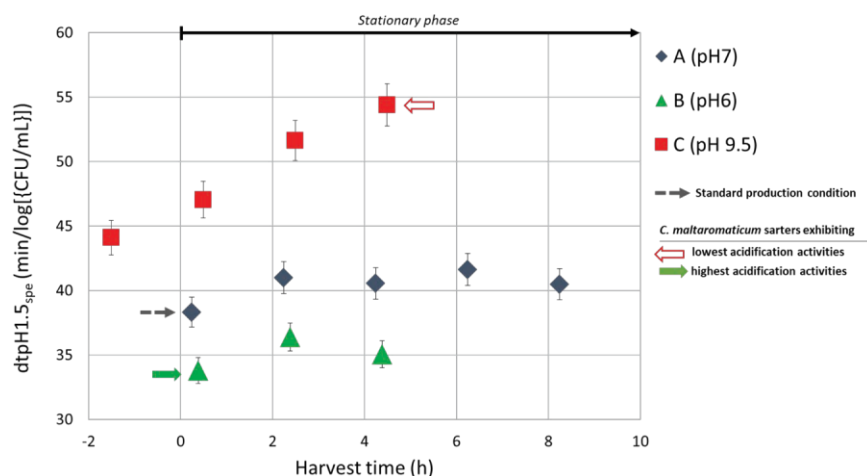
LAB industrial starter production generally involves a succession of steps from fermentation to stabilization processes (**figure 2**) and it is generally optimized to achieve the highest possible yield of concentrated cells exhibiting fast metabolic activities. In productions intended for TTI labels however, other objectives are pursued: production parameters are chosen according to the response time needed to be covered by the TTI label. For long shelf-lives, the starters inoculated into the label must indeed harbor slow acidification activities, while high acidification rates lead to labels exhibiting short shelf-lives. It is known that physiological state modulation of LAB is highly dependent on the fermentation parameters applied during their production (temperature, pH, fermentation duration, medium composition, *etc.*) (van de Guchte et al. 2002). The effect of culture conditions on the physiological state of some LAB species proposed for LAB-based TTI are presented in **table 4**.

**Table 4:** Effect of culture conditions on the physiological state of LAB proposed for LAB-based TTI.

LAB strains	Investigated culture conditions	Effect on physiological state	References
<i>Lactobacillus sakei</i> 23K	Fermentation at optimal growth temperature (26 °C) and upon reaching exponential phase: → Heat shock (HS): 20 min at 42 °C → Cold shock (CS): 20 min at 4 °C	- No difference in viability; - Both HS and CS induced better acidification activity; - Best acidification activity after CS.	Hüfner and Hertel 2008
<i>Carnobacterium divergens</i> V41	→ Temperature: 15 to 30 °C → pH: 6.5 to 8 → Carbohydrates (2 to 20 g L <sup>-1</sup> )	- Positive effect of low temperature and high carbohydrate concentration on viability; - No effect of tested pH range on viability; - Low pH increased bacteriocin production.	Brillet-Viel et al. 2016
<i>Carnobacterium maltaromaticum</i> CNCM I-3298	→ Temperature: 20 to 37 °C → pH: 6 to 9.5 → Harvest times: 0 to 10 hours into stationary phase	- No significant effect of fermentation conditions on viability of harvested cells; - Low temperatures and pH increased acidification activity of harvested cell concentrates; - High pH and extended harvest times decreased acidification activity of harvest cell concentrates.	Girardeau et al. 2019
<i>Lactobacillus rhamnosus</i> GG *	→ Temperature: 37 °C → pH: unregulated → Glucose (10 to 34 g L <sup>-1</sup> ) → Peptone (8 to 12 g L <sup>-1</sup> )	- Growth favored by high peptone concentrations; - Lactic acid production yields favored by high glucose concentration.	Mel et al. 2008
<i>Weissella cibaria</i> DBPZ1006 *	→ Temperature: 10 to 45 °C → pH: 4 to 8 → a <sub>w</sub> : 0.935 to 0.994	- Maximum growth rate achieved at 36.3 °C, pH 6.6 and a <sub>w</sub> of 0.994	Ricciardi et al. 2009

\*The results presented for these strains are the effect of culture conditions on the growth and metabolic activity of the cells during fermentation, not on the physiological state of the harvested cell concentrates.

Viable cell concentration and acidification activity are the technological properties that characterize the physiological state of cells for use in LAB-based TTI labels. The range of acidification activity and viability that can be covered by a strain of *C. maltaromaticum*, simply by changing three fermentation parameters (temperature, pH and harvest time), has recently been mapped (Girardeau et al. 2019). **Figure 3** shows the effect of fermentation pH and harvest time, the two most influential parameters on the biological activity of *C. maltaromaticum* concentrates characterized by their specific acidification activity (dtpH1.5<sub>spe</sub>, in min/log[{CFU/mL}]). Specific acidification activity is defined as the ratio of dtpH1.5 (the time necessary for the produced concentrates to acidify a TTI-like medium by 1.5 upH) to the corresponding log of cell concentration (colony forming unit [CFU]/mL). Consequently, the dtpH1.5<sub>spe</sub> descriptor gives an accurate and meaningful measurement of *C. maltaromaticum*'s physiological state, including its acidification activity and viability.



**Figure 3:** Specific acidification activity of *C. maltaromaticum* concentrates harvested at increasing times of fermentation, carried out at 30 °C and different pH values. Adapted from Girardeau et al. 2019.

*C. maltaromaticum* cells produced at low pH (e.g. pH 6) and harvested at the beginning of stationary phase exhibited faster acidification activities (solid arrow, **Fig. 3**) than cells produced in standard conditions (pH 7, 30 °C) (dashed arrow, Fig.3). These cells could potentially reduce the current lower limit of TopCryo®’s response time by two hours. Conversely, cells produced during fermentations carried out at high pH (e.g. pH 9.5) and harvested up to 10 hours into stationary phase (empty solid arrow, **Fig. 3**), exhibited slower acidification activities, thus potentially increasing TopCryo®’s upper limit response time by 68 hours. Although the effect of temperature was less pronounced than pH and harvest time, it was still significant: low temperatures (20 °C instead of the 30 °C of the reference condition) led to increased acidification activities, whatever the harvest time up to 10 hours of stationary phase. This study thus showed that identifying the range of acidification activities that can be achieved by a certain strain reveals the possible shelf-life range extensions that can be reached by TTI labels based on this strain. Following this approach for a given strain, once the temperature, pH and harvest time have been studied for identifying upper and lower limits of the range of acidification activities, further extension of the TTI’s response time range can be realized by tweaking the label medium composition.

It is important to note however, that the study on *C. maltaromaticum* CNCM I-3298 carried out by Girardeau et al. (2019) investigated the effect of fermentation parameters on the acidification activity of freeze-thawed cells, after confirming the strain’s high resistance to freezing, even when produced at extreme fermentation conditions (i.e. pH 6, 20 °C or pH 9.5, 30 °C). Such resistance to freezing is very much dependent on the considered LAB genus, specie or even strain. Freezing or freeze-drying exposes bacterial cells to big changes in environmental conditions like low temperatures, high solute concentration and extreme dehydration (Meneghel et al. 2017). Bacteria passively respond to stresses leading to cellular injuries and rather severe deterioration of biological functionalities after freeze-thaw or rehydration. It has been shown that LAB resistance to different stresses is also greatly dependent on their fermentation parameters (Palmfeldt and Hahn-Hägerdal 2000, Wang et al. 2005, Velly et al. 2014). During fermentation, stress proteins can be synthesized and membrane lipid compositions can be changed (Schoug et al. 2008). Promoting active cell response to moderate stressors during fermentation can help minimize the effect of

subsequent stressors that cells face during stabilization steps. The use of protective solutions containing various substances such as polyols, polysaccharides and disaccharides has also been shown to help lessen cellular damage and improve storage stability (Linders et al. 1997, Conrad et al. 2000, Reddy et al. 2009). In LAB-based TTI production (Fig. 2), cells undergo two stabilization/activation cycles: the first one after fermentation and the second one within the TTI label. It is therefore all the more crucial to check that cells maintain their viability and acidification activity between the moment they are harvested after fermentation to the moment they are reactivated within the TTI label. The resistance to stabilization of potential strains for TTI use must thus be thoroughly investigated and optimized.

#### *Tweaking the label medium's composition*

*pH and chromatic indicators.* The choice of chromatic indicator contained within a label greatly influences the time it takes for the color shift to take place. In TopCryo® labels for example, the chromatic pH indicator shifts from bright green at pH 8 to dark red at pH 6.5. In Vaikousi's proposed prototype (Vaikousi et al. 2008), the pH indicator is chlorophenol red, shifting from red at pH 6.1 to yellow at pH 5.2. In both TTI, the response time is thus dependent on the time it takes for the microorganisms to acidify the label medium down to the pH value at which a distinct visual color can be observed. Color shift response times can thus be modulated by either increasing or decreasing the difference between initial and final pH.

*Nutrient availability.* Nutrient availability within the TTI label medium could also be a response time modulation lever. Indeed, lactic acid bacteria are known to have many nutritional requirements and their production often demands growth in nutritionally rich environments. Most nutritional studies performed on LAB are focused on maximizing growth rates and/or metabolic activity. In the case of a TTI application, research on nutritional levers that either accelerate, or slow down metabolic activity are both relevant when developing large range of shelf-life response times. LAB growth requires first and foremost the presence of a carbon source through carbohydrates, their main energy source. The nature of the carbohydrates (monosaccharides, disaccharides, pentoses, hexitols or penitols) varies according to the considered species or strain and sometimes even between strains. It is frequent for a strain to be able to catabolize more than one type of carbohydrate, but its growth and metabolic kinetics will change depending on the available type. In a study aimed at investigating the growth and metabolic performances of *L. sakei* strains on different carbon sources, it was shown that using ribose as a sole carbon source decreased growth and increased acetate production compared to glucose (McLeod et al. 2010). Changing the nature of the carbon source within the TTI label medium can thus have an impact on the growth and/or acidification activity rates of the selected strain, therefore resulting in the adjustment of the TTI's response time. Nutritional studies have also shown that LAB growth can be stimulated by the addition of ingredients rich in amino acids and small peptides, such as yeast extract or peptone. *C. maltaromaticum* for example exhibits substantially better growth in milk when it is enriched with 1 g L<sup>-1</sup> of yeast extract (Edima et al. 2008). Similarly, certain vitamins and nitrogenous bases were shown to stimulate growth, although requirements vary greatly even between strains of the same species. This was clearly evidenced in a study aimed at screening for vitamin and amino acid requirements on 24 *Lactobacillus plantarum* strains, where a very high diversity of requirements between strains was demonstrated (Ruiz-Barba and Jiménez-Díaz 1994). Even if generally time consuming, great potential for TTI shelf life modulation can thus arise from modifying nutrient availability in the TTI medium.

*Water activity.* One of the oldest methods of preventing food spoilage is drying. Its direct consequence is to reduce water activity ( $a_w$ ), thus limiting the available water required for the growth of microorganisms. Adjusting the  $a_w$  of an aqueous growth medium is often done by the addition of salts, saccharides or polyols. Growth rate and maximum viability reductions, as well as acidification activity modulations are observed in LAB when  $a_w$  levels are reduced from 0.99 through 0.95 or 0.93, depending on the species and compounds used to lower water activity (Ruiz-Barba and Jiménez-Díaz 1994). Interestingly, the effect of  $a_w$  on growth and viability can also be linked to temperature, pH and available nutrients. Such a link was for example evidenced in a study of the effect of suboptimal temperatures and high salt concentrations on the growth and long-term survival of *Lactobacillus sakei* (Marceau et al. 2002). Incubation at 4 °C or in a 9 % NaCl solution respectively resulted in *Lb. sakei* survival of 16 and 10 days. However, when cells were incubated at 4 °C in a 9 % NaCl solution, cell viability was stable between 24 to 28 weeks. The relationship between temperature and water activity, and its impact on the kinetic of cell development and acidification are thus particularly relevant in TTI research.

Tweaking a TTI's medium composition therefore offers many possibilities to modulate a biological TTI's shelf-life response time and therefore widen the shelf-life range that can be achieved by a single strain. There are however various technological and design constraints linked to label production at industrial scale that must be taken into account, such as viscosity, cost, consumer behavior, *etc.*

#### *Using different bacterial species or strains*

In addition to being dependent on initial cell concentrations, TTI response time evidently relies on the inherent growth and acidification kinetics of the selected lactic acid bacteria. Another strategy to widen the covered range of shelf-life is to switch bacterial species entirely. Due to their diversity, lactic acid bacteria exhibit very different growth and acidification kinetics, depending on the considered genus, species or strain, on whether they are homo or hetero-fermentative and on their culture conditions.

Homofermentative LAB, such as *Lactobacillus bulgaricus* or *Lactococcus lactis*, are widely used in the dairy industry because they exclusively metabolize sugars into lactic acid ( $pK_a = 3.86$ ), resulting in high acidification activities. Heterofermentative LAB however, tend to have much slower acidification activities because their fermentation of sugars results in numerous other metabolites, including acids that might not have the acidification power of lactic acid, such as acetic acid ( $pK_a = 4.76$ ), butyric acid ( $pK_a = 4.82$ ) or propionic acid ( $pK_a = 4.88$ ) and ethanol. Proportions among different produced metabolites may also vary depending on the microorganism and applied fermentation parameters. A good example of a slow acidifying, heterofermentative LAB is *Carnobacterium maltaromaticum*, the strain contained within Cryolog labels.

LAB used in biological TTI must follow certain conditions. They need to grow within the temperature range perishable foods are stored at (typically between 0 and 30 °C) and their growth and metabolic activity must be highly dependent on temperature, allowing an accurate monitoring and recording of all temperature variations throughout the product's shelf-life. Additionally, growth must not be inhibited by the pH-indicator used to indicate end of shelf-life. Furthermore, most LAB-based TTI developed to date use psychrotrophic strains that present growth rates similar (or slightly higher) to those of main pathogenic and spoilage bacteria contained within the food being tracked (*e.g. Listeria monocytogenes*). This way, the TTI endpoint is attained before the growth of those microorganisms reaches unacceptable levels.

*C. maltaromaticum* follows all the above conditions needed to be an adequate micro-organism for use in TTI: it is a psychrotrophic lactic acid bacteria commonly found in many highly perishable, high protein foods such as meat, fish and shellfish, and it has sometimes been regarded as a spoilage bacterium, although non-pathogenic. *C. maltaromaticum* was initially not considered to be a LAB species of industrial interest due to its slow acidification activity compared to other starter LAB such as *Lactococcus lactis* or *Streptococcus thermophilus* (Edima et al. 2008). However, since it has been regarded as a good candidate for biological TTI, its kinetic properties have been extensively studied and several growth and acidification models were developed to predict its behavior in different TTI labels offered by Cryolog (Ellouze et al. 2008; Girardeau et al. 2019). Other LAB strains that have been proposed in biological TTI systems are *Lactobacillus sakei*, *Lactobacillus rhamnosus* and *Weisella cibaria* (table 2), all LAB commonly used in the food industry. *Lactobacillus sakei* is a heterofermentative psychrotrophic lactic acid bacterium used in various fermented meats such as *saucisson* (French dry cured sausage). This strain presents similar kinetic behaviors as many spoilage bacteria of meat products (Vaikousi et al. 2009) and has in addition been shown to inhibit *Listeria monocytogenes* in chicken cold cuts (Katla et al. 2002). *Lactobacillus rhamnosus* and *Weisella cibaria* are both heterofermentative, mesophilic bacteria. The first is known for its probiotic properties (Saad et al. 2013) and the former is commonly isolated from fermented foods and increasingly being investigated to be used as a starter for sourdough and vegetable fermentation (Ricciardi et al. 2009).

An additional approach to further extend TTI response ranges would be to investigate the use of co-cultures. Bacterial cultures applied in food fermentations mostly consist in consortia involving multiple species and/or strains, where each member is affected by the growth and metabolism of the other. A perfect example of this is the yoghurt consortium, consisting of a proto-cooperation between *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. In milk fermentation to produce yoghurt, the first microbial exponential growth phase is the one of *S. thermophilus*, a species that is more tolerant to neutral pH and more effective at metabolizing amino acids and trace elements than *L. bulgaricus* (Sieuwert, 2016). The growth of *S. thermophilus* results in medium acidification, reduction of oxygen and increase of CO<sub>2</sub> concentrations, thereby creating a favorable environment for the more acidophilic, less oxygen tolerant *L. bulgaricus* (Sasaki et al. 2014). A similar type of interaction could be imagined within a biological TTI, where a strain such as *C. maltaromaticum*, very tolerant of high pH values (up to pH 9.5), would start acidifying the TTI medium down to a value that favors the growth of a second, more acid tolerant strain such as *W. cibaria*. The combination potential between two or more microorganisms within a TTI is immense, as microbial interactions are governed by a multitude of molecular and physiological mechanisms. Still, systematic optimization and repeatability studies are required to choose and confirm the applicability of new bacterial species in a biological TTI system. Moreover, the process of acquiring a take-to-market authorization for a single or a combination of species are both long and expensive. Mapping the range of shelf-lives that can be achieved with a selected strain as well as screening new strains and co-culture exploration must therefore be concurrent R&D activities.

#### *Concluding summary on LAB-based TTI parametrization*

LAB-based TTI parametrization can be done according to different strategies. For a given micro-organism(s) they include: adjusting the initial starter concentration, modulating the physiological state of a selected strain or tweaking the TTI's core medium by adjusting its chemistry (initial pH,  $a_w$ , available

nutrients...), and switching chromatic pH indicators. Selecting different micro-organisms (genre, species or strain), in pure cultures or in associations (co-cultures) are also relevant approaches.

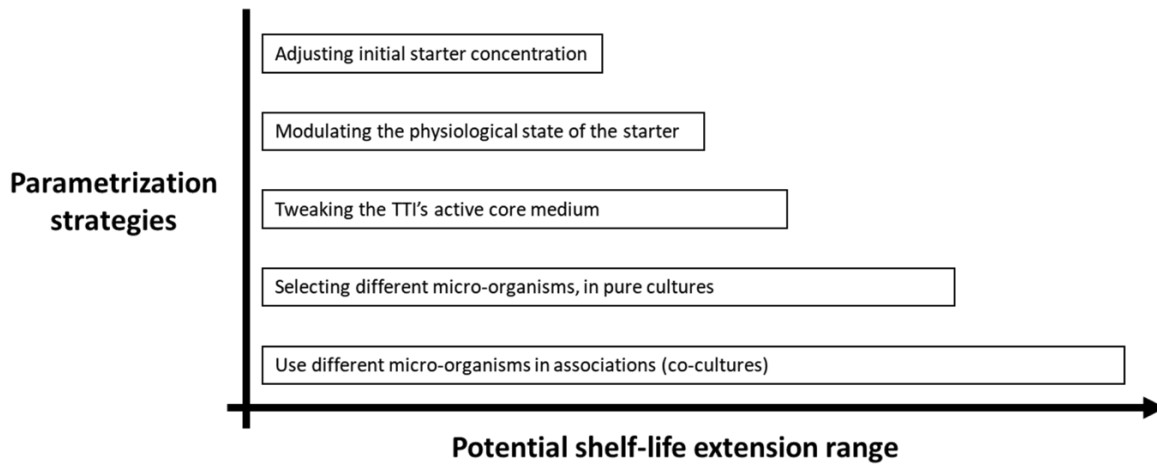


Figure 4: Schematic representation of biological TTI parametrization strategies and their expected potential shelf-life extension ranges.

Figure 4 summarizes the different parametrization strategies described in this chapter and their corresponding potential in extending the shelf-life ranges covered by a biological TTI device. All of these strategies can be pursued sequentially or in parallel to achieve response times covering the widest possible range of perishables food shelf-lives, from shellfish to sandwiches.

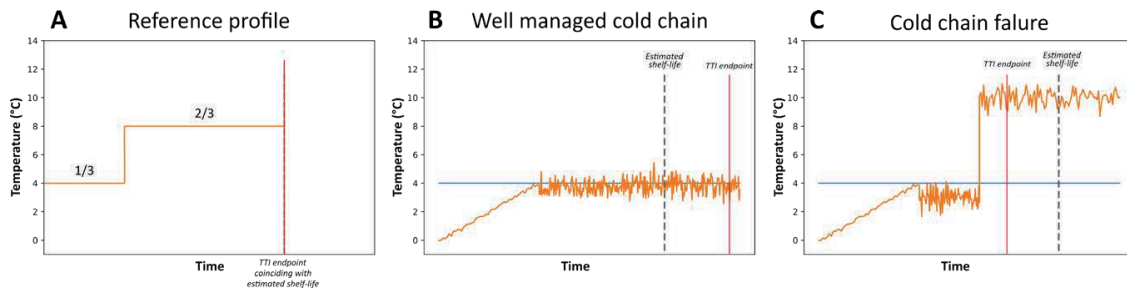
### Performance evaluation

In many of the studies proposing biological TTI prototypes (table 2), the Arrhenius activation energy ( $E_a$ ) indicating temperature sensitivity of the TTI's response is used as the main performance evaluation parameter (Vaikousi et al. 2009, Kim et al. 2012, Lim et al. 2014). According to this approach, a TTI is considered performant if it presents similar  $E_a$  response values as the spoilage reaction being tracked. However, relying solely on activation energy values is not sufficient to prove the applicability of a system intended to track the microbiological quality of specific perishable foods. In order to properly evaluate performance and be granted a take-to-market authorization, a LAB-based TTI must undergo similarly rigorous testing to shelf-life date labels: durability studies and challenge tests.

First, food business operators generally start by consulting predictive microbiological models to estimate the microbiological quality evolution of a product. While these models provide good estimations of spoilage kinetics by taking into account composition and manufacturing processes, they are developed assuming consistent microbial responses in very controlled environments and can thus sometimes fail to accurately predict inconsistent microbial responses and variations in a complex food matrix. This first shelf-life estimation must therefore be followed by durability studies. Durability studies (or storage tests) are conducted by quantitatively determining growth of target spoilage and pathogenic microorganisms within their food product, under “reasonably foreseeable conditions of distribution, storage and use” (European Commission Regulation No. 2073/2005). A recommended temperature scenario or reference profile is to maintain the food product one third of its estimated shelf-life time at 4 °C and two thirds at 8 °C. For highly

perishable foods that may contain pathogens such as *Listeria monocytogenes*, EU guidelines recommend additional challenge tests. Challenge tests are similar to the durability studies mentioned above, with the difference being that specific cold-tolerant pathogens are added to the food product before it is packaged. This is especially important when the microorganism's environment within the food product (chemical characteristics, temperature,  $a_w$ , etc.) is close to its growth/no growth limit.

The applicability of Cryolog's commercialized TTI was therefore evaluated with both durability studies and challenge tests using ground beef, cooked chicken and smoked salmon (Ellouze et al. 2010, 2011). These food products and their corresponding TTI labels were exposed to three dynamic temperature profiles (Fig. 5):



**Figure 5:** Simulation of different, realistic storage conditions used to evaluate LAB-based TTI performance.

- A. A reference profile that was used to estimate shelf-life: one third of the time at 4 °C and the rest of the time at 8 °C;
- B. A well-managed cold chain scenario with temperature fluctuations averaging below the reference condition (4°C);
- C. A cold-chain failure scenario with temperature fluctuations averaging well above reference condition.

For each temperature profile, the growth of endogenous food microorganisms and added pathogens was evaluated at the end of the estimated shelf-life and at the TTI's endpoint (Ellouze et al. 2010, 2011). Results showed that for the first profile (reference profile, Fig. 5A), the TTI endpoint coincided with the estimated shelf-life, confirming proper TTI parametrization according to current established guidelines within the food industry. For the second temperature profile (well managed cold chain, Fig. 5B), the TTI endpoint came after the estimated shelf-life, but still before pathogenic or spoilage microorganism levels reached consumption limits. This scenario thus demonstrates how TTI can contribute to reducing food waste. In the third profile (cold-chain failure, Fig. 5C), the TTI endpoint was reached before the estimated shelf-life, thus evidencing how the use of TTI helps avoid consumption of potentially spoiled food. Cryolog thus successfully proved their TTI system's ability to integrate the time-temperature history of highly perishable foods and adjust shelf-life information accordingly. Smart devices such as these can thus contribute to reducing food waste (Fig. 5B), without compromising food safety (Fig. 5C).



## Current applications and prospects

Research on various time-temperature integrator devices increased back in the early 1980s, in the hopes of replacing shelf-life date labels entirely, with a label that was more representative of the real time-temperature history of a product and also more comprehensible by consumers (Blixt and Tiru 1976; Labuza 1982; Taoukis and Labuza 1989). Half a decade later, although food regulations in most countries still currently require a date on shelf-life labelling of pre-packaged perishable products, an increasing number of food business operators use TTI, along specific stages of their manufacturing process to control proper cold-chain adherence and raise staff awareness.

A good example of this kind of TTI usage, is that of an industrial catering kitchen using TopCryo® labels to ensure cold-chain integrity of products that go through preliminary preparations before packaging. Following a preliminary hazard analysis, the kitchen's quality control manager determined that products undergoing a preliminary preparation phase, such as slicing, must then be stored at a maximum temperature of 3 °C and used within three days. Before using TTI labels, if one of those intermediary products had been temporarily exposed to temperatures above 3 °C the established precautionary principle at that company was to either use it within 24 hours or discard it. Since using TopCryo® label parametrized for a three-day / 3 °C shelf-life, rules have changed and produce are safely used within the three-day limit, as long as their TTI label remains green. Another example of current LAB-based TTI use is in the deli section of a supermarket, where the sanitary control plan established an internal shelf life of four days at 4 °C for all sliced meats. During a test phase, corresponding TTI labels placed next to the meats met their endpoint under four days in more than half of the cases, highlighting poor cold-chain management (display temperature too high, long waiting time between cutting and refrigeration). The quality control manager was then able to bring awareness to the staff and implement corrective actions.

LAB-based TTI are also currently making their way onto catering trays, take-away boxes and food delivery packages to inform customers of proper storage and transportation within the cold-chain.

As TTI research continues to grow, LAB-based TTI labels will certainly become an inherent part of production, storage, transportation and distribution of perishable food products. Supermarkets could add TTI tracking devices on their drive-through grocery pickup or delivery bags and postal services could paste TTI labels on temperature sensitive packages. Ultimately, TTI information such as color/pH recognition could be digitally integrated and used on mobile apps, allowing remaining shelf-life calculations, data collection, *etc.*

## Concluding remarks

Time-temperature integrators are devices that are not meant to simply report a temperature limit breach but to translate all inevitable temperature variations experienced by perishable foods, from production to distribution. They thus integrate the time and temperature history of the product they are tracking and adjust its shelf-life information accordingly. Among the different types of TTI systems developed to date, biological TTI have the advantage of basing their response upon the same, temperature dependent, microbial growth and metabolic activity that causes food spoilage and end of shelf-life. To date, the biological TTI system on the market offers a limited range of shelf-life. A lot of work has yet to be carried out in biological TTI research and in overcoming regulatory obstacles that keep this technology from being fully deployed within the food industry. Nevertheless, it is a promising and reliable technology that has great potential in reducing food poisoning risks and food waste.

## Acknowledgments

This work was supported by Clock-°T (Cryolog). This project has also received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 777657.

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