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# **A Whole New Generation of Milk Kefir Grains Formed From Freeze-dried Starter Cultures A Fascinating Insight Into a Hidden World**

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**KEY WORDS:** Kefir grains; symbiosis; milk fermentation; starter-cultures; bacteria; yeasts

## **ABSTRACT**

Milk kefir grains (KGs) are composed of a complex symbiotic microbial mixture of bacteria and yeasts growing in kefiran, an extracellular polysaccharide substance of bacterial origin. This is the first report documenting a successful attempt at reforming KGs using pure mixed starter-cultures of microorganisms isolated from KGs, and gain insight into an otherwise hidden world. A milk pouch system designed to concentrate the starter-cultures for 72 h, made this transformation possible. The smaller granules, typically less than 1 mm, have blossomed into a reliable mother-starter and have been reproducing by daily transfers for over three years transforming milk into a delicious beverage. The larger grains, up to about 2 cm (N=12) were considered adequate material for further studies on KGs because each grain had a unique design that could potentially be tracked. They were maintained during repeated cycles for up to four months in a semi-transparent medium enriched with fresh grains which allowed close examination of their morphological features and collective motions in amazing new detail, not otherwise visible in milk. The grains are continually in motion, presumably in search of symbiotic relationships. The data show that clusters of biomass are continually released through cracks and splits, sometimes liberating new 'baby-grains' from the 'mother grains'. Together, they form an intriguing pattern of dynamic grouping suggestive of pair-bonding that have not been visualized, up till now. It can be concluded that a whole new generation of KGs has been created. The original starter cultures became encapsulated in an entirely natural form. It is likely that natural airborne bacteria and yeasts took over at some point to produce symbiotic communities closely interacting together, and able to survive and reproduce. There is still much to discover about the natural behaviors of these amazing creatures.

## INTRODUCTION

The increasing popularity of fermented milk products comes from the fact that they have well recognized nutritional benefits which have been the subject of extensive research all over the world. People from many countries are consuming kefir-type products every day, some of which are being made of milk kefir grains (KGs) also called 'Tibetan Mushrooms', 'Grains of the Prophet Mohamed' and 'Snow Lotus'. Those grains consist of discrete clusters of bacteria and yeasts existing in symbiotic association, and encapsulated within a self-produced matrix of extracellular polymeric substance (EPS) of bacterial origin. Each grain represents an amazingly diverse ecosystem. The many stories surrounding their origin have fascinated humans for centuries as reviewed in [1-3]. The potential health benefits from consuming KGs regularly are well documented. In particular, their regulatory functions on the immune system and other tissue organs have been described in the literature but are beyond the scope of this paper [4-7].

There are various methods available for the preparation of milk kefir throughout the world. Kefir can be made using microbial isolates prepared as a freeze-dried starter and/or lyophilized grains but it is more palatable and nutritious when fresh grains are used as a starter for the milk culture [4-7]. The large-scale production of kefir involves a two-step fermentation process. The first fermentation is achieved by adding KGs to pasteurized milk (2-10%, w/v). After a period of fermentation lasting around 24 h, the grains are removed and the filtrate is added to milk (1-3%, v/v), which is further fermented for 24 h and packaged for the consumer market. Plain kefir can be found in pasteurized forms in many stores; it contains a few select strains of bacteria and yeasts, whereas traditional kefir produced at home, contain a plethora of natural strains (>60) and many other compounds occurring naturally during fermentation from the live grains. A detailed description of the typical strains most commonly found in traditional kefir is given in references [8-13]. Bacterial species are mainly of the genus lactobacilli, but also include members of the genera lactococci, streptococci, and leuconostoc. The yeast species mainly of the genera Kluyveromyces, Candida and Saccharomyces occur in this ecological niche.

Morphologically, the grains are characterized by irregular clusters of bacteria and yeasts, and sometimes molds, forming symbiotic communities [14-17]. The grains are white to off-white and when placed in milk, they swell. The most active grains float near the surface, and store a reservoir of nutritious fluid in their hollow interior. They tend to clump together and expand to a varying extent. Over time, CO<sub>2</sub> is released from the grains in discrete bursts.

Photographs of grains collected from all over the world have been published [18, 20-22]. When maintained in continuous culture, the grains can propagate indefinitely as long as they are given fresh milk on a regular basis. They need constant care and have the potential to become more reliable from repeated use. Because of their natural resilience, these cultures have the potential to last for a lifetime and beyond.

The microbial species are mutually dependent and must remain in balanced proportions inside the grains to ensure that the resulting kefir is palatable and nutritious. The yeast component is essential, although its proportion is much lower than that of bacteria (~30% and 80%, from reference [23]).

The nutritional value of kefir is based on the use of milk as a main ingredient and the subsequent changes that occur as a result of primary and secondary fermentation. In addition to the mode of handling, the presence of the grains and their fermentation properties will, in the end, determine the quality of the kefir obtained. Primary fermentation occurs at room temperature (18-23°C) during which time there is consumption of lactose, and production of heat energy, water, CO<sub>2</sub>, organic acids and ethanol along with many other compounds including a smooth gel from the EPS-producing strains of bacteria. The best texture and flavor develop during the secondary fermentation when the prepared kefir is chilled for several days. During this time, the fermentation continues and secondary compounds including aromatic substances are formed by the growing cultures through microbial enzymatic activity. Some metabolic products are volatile while others may be assimilated further, contributing to the delicate texture and flavor combined with a slight fizzy character of authentic kefir [1-5].

Much of the knowledge about KG composition has come from culture experiments on component strains isolated from KGs that are being used to prepare kefir starters. Commercial starters based on isolated microbial components have limited usage and will not produce real kefir [10]. To make authentic kefir, one needs access to KGs. These grains are uniquely designed to create a natural habitat for a wide diversity of beneficial strains acting symbiotically [23-26]. Once the strains are isolated from their natural habitat, some of their properties are lost [6-7].

No one has yet succeeded in reconstructing KGs from pure mixed starter-cultures of microorganisms isolated from KGs or crude cultures [5, 22, 27-28]. Despite unsuccessful attempts by others, the author contemplated the possibility of using component strains as starting material with the aim of producing a whole new generation of KGs. If feasible, it would be interesting if one could get a glimpse into their hidden world and get close enough to see what the grains really look like in situ, and explore their natural behaviors.

The project started in the summer of year 2012. The aims of this research were 1) to provide direct experimental evidence that it is possible to reconstruct KGs out of microbial isolates using a milk pouch system to concentrate the starter-cultures; 2) to begin a milk culture using the small granules (newly discovered) as a mother-starter, and 3) to study the larger grain specimens from the same source in an experimental culture model (ECM) system that would open a window into the world of KGs and provide close view of their natural behavior and possible interactions. During recording, it was essential to minimize external vibrations/turbulence so that collective motions and any particular behavior would be monitored without external interferences.

Watching the way the grains move, transform, and interact – presumably in search of symbiotic relationships – was fascinating and has left an indelible impression on the observer. There is no doubt that the grains can communicate and these interactions are a vital part of how they are able to share resources, adapt and survive in their own world. Ultimately, this work was aimed at contributing to the existing knowledge on KGs as part of a complex and diverse microbial ecosystem.

## **MATERIALS AND METHODS**

**Supplies.** All equipments used were made of food-grade material. Clear glass jars (dimension: 9.5 x 7.0 cm; capacity: 300 ml) were purchased from Euro Cuisine Inc. (Commerce, California, USA). Frigoverre glass pitcher (dimension: 15.0 x 7.0 cm; capacity: 750 ml) was purchased from Bormioli Rocco Glass Co (Fidenza, Italy). Food storage container with lid, 9.5 liter-capacity (serving as ‘microclimate’ chamber) was purchased from Rubbermaid Inc. (Huntersville, North Carolina, USA). Other containers of various sizes were used for batch cultures as needed. Disposable plastic spoons were purchased at a local store. Ultra fine cotton cheese cloth (2.0 cm x 3.0 cm) and butcher’s twine were purchased from Regency Wraps, Inc. (Dallas, Texas, USA). A set of nylon mesh strainers (dimension 14-cm; mesh size = 0.8 mm) was purchased from Harold Import Co Inc. (Lakewood, New Jersey).

**Ingredients.** Grade A pasteurized homogenized cow’s milk with vitamin D, and instant nonfat milk with vitamins A and D were purchased from Jerseymaid Milk Products, Inc. (Vernon, California, USA) and Carnation Nestle SA. (Vevey, Switzerland), respectively. Organic brown sugars of various sources were purchased at a local health-food store, and consisted of evaporated sugar cane juice, coconut palm sugar, blackstrap molasses (a viscous byproduct of sugar cane and a good source of iron, potassium, calcium and magnesium), and candi sugar crystals derived from beets. Organic coconut water, Baker’s yeast, Japanese green tea (parts used: whole leaves, root, stem, and bark), dried fruits (figs, dates, raisins, cranberries) and fresh fruits (bananas) were purchased at a local store. Freeze-dried kefir starter containing microbial isolates in powder form, was purchased from Lyo-San Inc. (La Chute, Quebec, Canada). Each packet of five grams contains skim milk powder and ascorbic acid as cryoprotectants, as well as at least 3 billion lactic bacteria and 50,000 yeasts (cell count per gram). According to the manufacturer’s product information, the major select strains in the kefir prepared from this starter, are *Streptococcus Lactis*, *Streptococcus diacetylactis*, *Streptococcus cremoris*, *Lactobacillus casei*, *Lactobacillus acidophilus* along with yeast strains such as *Saccharomyces lactis* and *Saccharomyces cerevisiae*. In addition, there may be other bacterial species (10–20) derived from the original grains used to prepare the starter [29]. Brewer’s yeast suspension

(*Saccharomyces cerevisiae*, California Ale yeast, WLP001 ale yeast)) was purchased from White Labs, Inc. (San Diego, California, USA) [30]. Yeast supplementation is known to stimulate growth of KG cultures [31-41].

## **Methods**

### **General**

This is not a classical experiment in a laboratory setting. All cultures were kept at room temperature (28 to 30°C) and relative humidity (70 to 75 %), in a private kitchen area, under semi-controlled conditions. Because the cultures are sensitive to a number of experimental manipulations and changes in microbial populations induced by environmental conditions, considerable caution was exercised to minimize the potential risk of contamination during the preparation of the kefir beverage [7]. This work was carried out by a single person (the author) who was solely responsible for all phases of the study thereby limiting handling of KGs. Full precaution was taken to protect the cultures at all times from dust and any other potential interference, while allowing for natural air currents to take place.

It should be noted that the author used a blinded approach having no prior knowledge of KG cultures and therefore no expectation of what the outcome should be. In other words, the author had no pre-existing beliefs that could have altered the interpretation of results.

### **Reconstruction experiments**

The experiment was conducted in two phases. In the preliminary phase, plain kefir was produced using microbial isolates prepared as a freeze-dried starter [29]. According to the manufacturer's instructions, a packet of 5 grams of freeze-dried powder makes 1 liter of kefir. Pasteurized milk is heated up to 82°C and cooled down to room temperature. The starter powder cultures are stirred in milk and left to incubate over 24 h at room temperature (28 to 30°C). Kefir from the previous batch (up to 10%, v/v) is added to start a new batch. With this method, kefir is palatable with a smooth texture resembling plain yogurt/buttermilk and varying degrees of whey separation. These starter cultures have limited usage because they do not contain KGs. Only three or four batches could be made out of a packet of 5 grams which prompted us to continue the investigation.

In an attempt to make KGs come alive, two packets (10 grams) were concentrated in a home-made pouch, packed together with candi sugar crystals (15 grams) as a source of fermentative sugars. The pouch was securely tied up in place, and then, partially immersed in pasteurized milk (1 liter), previously heat-treated as shown above. The same pouch was repeatedly transferred to fresh milk, once daily over 72 h. At each transfer, fermented milk was added (10%, v/v) to boost

the next fermentation cycle. The experiment was carried out in triplicate. After the last 24-hour incubation period, the 'granular material' discovered within each pouch was collected in a fine-mesh strainer, and suspended in fermented whey (from above) before being subdivided into two distinct groups, based on size. The smaller granules were used to prepare kefir based on a traditional method, whereas the larger grain particulates were tracked separately in an ECM model described in the following sections.

### **Preparation of kefir based on traditional method**

The production of authentic kefir involves a two-step fermentation process [7]. In this work, the milk culture began by using the smaller grains (newly discovered) as a mother starter and maintained by the method shown below.

#### Primary fermentation

At the commencement of fermentation, add a tablespoon of KGs to 250 ml of cold milk. Do not fill completely to allow room for expansion. Stir briefly, once or twice. Place in a large 'microclimate' chamber for 24 h, and let the culture ferment, ideally at 20-25°C, agitating once or more times a day, so the cultures and nutrients are evenly blended. The culture itself is not covered as it needs to breathe. When agitated, some CO<sub>2</sub> bubbles will come through and one can hear a fizzing sound. Over time, the milk will gradually thicken and more grains will emerge near the surface. Each time, a portion of the fermented medium (10%, v/v) is left to boost the next cycle and a tablespoon of active grains is used as the starter for the same amount of fresh milk in the next batch. The same procedure is repeated over and over again, producing a regular supply of fresh grains. To scale up the procedure, the author has found that two or three small batches are better than one large one as it allows more control over the fermentation.

The grains need constant care. As long as they are given fresh milk and a fraction of the grains are transferable repeatedly (daily), the culture should be able to survive indefinitely. The best conditions for grain propagation to occur require a number of variables to be achieved, involving temperature gradients, relative humidity, periodic agitation and sufficient nutrients to support overall growth of the different species. The microorganisms help each other to maintain a balanced life. They are mutually dependent and grow in balance proportion which translates into more aroma, flavor and texture to the finished kefir.

### Secondary fermentation

After the primary fermentation is over, the next step is to proceed with the secondary fermentation to improve flavor and texture. In the later stage of fermentation, the acidified milk (fresh kefir) is chilled at 8-10°C, loosely covered with plastic wrap to let excess CO<sub>2</sub> escape. This will create varying degrees of effervescence depending on storage conditions. After several days of cold storage, the fermentation is considered complete.

Ideally fresh kefir equal in bulk to the portion removed is added and this is done continuously. Blending fresh kefir to kefir standing in the fridge on a regular basis will create greater complexities of flavor and texture due to carry-over effects.

### Grain preservation protocol

Preserving the grains is recommended to ensure continuity of the cultures in case the original grains are lost or damaged [7]. The freshly-made grains can be stored for several days at -4°C. They can remain in the prepared kefir sitting in the fridge or be removed and stored separately in a small container, covered with milk. For long term storage, the grains to be preserved are partially dehydrated on a clean paper towel and flipped regularly over several hours to accelerate drying. When the paper towel looks dry, this is an indication that the grains are ready to be tossed in dry milk powder. By using this procedure, the grains have been found viable when stored for up to 18 months at -20°C.

### Grain recovery procedure

A new rapid recovery procedure was used to restore integrity of the grain cultures. Grains are influenced by surrounding conditions. They can only be retained in their best conditions for a limited time through the exercise of the greatest care. Some conditions have a distinct influence on diminishing the size of KGs particularly, dry and cold weather. When it has become much more difficult to cultivate the grains to a point that they are extremely minute and in very low numbers after straining, it is recommended to simply adjust the amount of fresh milk in 25 ml-increment every 24 h to give a chance for the grains to grow back and ramp up their production. It is also possible to increase their biomass and 'resurrect' them in just a few steps.

**Step 1:** A liquid yeast extract is prepared according to [7] with the following modifications. The 'fermentation medium' described in the next section, is enriched with organic coconut water added in equal amount to fermented whey liquid with approximately 20% w/v of supplemental ingredients such as a few dried cranberries, green tea and a pinch of Baker's yeasts (*S cerevisiae*). Be sure to stir or shake well to get enough dissolved oxygen. Leave at room temperature for 12 h, stirring once or more times, and chill for a few days until use.



Step 2: Combine liquid yeast extract (supernatant) with an equal amount of milk kefir – derived from first and/or second fermentation – in a tall glass, stirring a few times during the day. The culture is exposed to the surrounding open air to allow natural wild yeasts and bacteria to inoculate the cultures. Overnight, the grains will rise to the surface, nestled among very a thick surface foam layer presumably containing top-fermenting yeast activity. The grains are half-hidden by the foam and not distinctively recognizable.

Step 3: The foamy mass is directly scooped out to be placed in milk. Beyond this stage, the culture can proceed normally based on the traditional method described above. The newly-emerging grains are golden-brown and will quickly turn white-to-off white after repeated passage in milk.

### **Experimental model**

A new ECM system was developed to detect morphological features and collective motions otherwise not visible in milk. The culture began by using the larger grain specimens (N=12) as initial inoculum. A detailed description of the culturing process is provided below:

The whey-molasses solution was prepared as follows: 500 ml of 1% molasses solution was made freshly on each day by combining 5 ml of molasses with 450 ml of filtered water and fermented whey from the milk culture (1:1, v/v). The solution was used as fermentation medium with 15 % sugar content (w/v). A 100- $\mu$ l suspension ( $\sim 1 \times 10^9$  cells) of Brewer's yeasts was added during the third week to boost yeast content, and potentially stimulate growth of KG cultures [31-41].

Fresh supplemental grains derived from the milk culture were added twice weekly to enrich the medium. The medium was also supplemented with a variety of fruits every other week. The grains were repeatedly transferred, and given fresh fermentation medium at 24-hour intervals, for four months. Between each cycle, the grains were visually inspected and photographed, before being returned to fresh medium. Each grain was turned over manually to see what the other side looks like. Small grain debris resembling fine sand materials were excluded.

Each time, a portion of the fermented medium (10%, v/v) was left to boost the next cycle. The unused fermented medium was transferred to a storage container and chilled of a few days in the same manner as described earlier, before being consumed. Cold storage for several days resulted in a fizzy, cloudy beverage that is low acid, somewhat sweet and slightly alcoholic. The flavor and aroma of 'water kefir' produced in this manner is very complex, being derived from a vast array of components that arise from a number of sources. The most notable of these byproducts are curd fragments, ethanol and CO<sub>2</sub>; but in addition, a large number of other flavor compounds are produced such as fruity esters (flavor and aroma of bananas, figs, cranberries, or other fruits).

The culturing process was carefully monitored through repetitive fermentation cycles, to prevent nutrient deprivation due to high cell density and accumulation of acidic products. Sensory measures were used to get an estimation of the metabolic demand of the grains in culture. This is important since an imbalance can cause the viability of the KGs to decrease quickly. A medium that has become significantly depleted of nutrients has a distinct yellowish color and cloudy appearance due to high cell density that once observed, is easily identifiable. Also, sweetness is one of major sensory determinants, and one will notice a change in sweetness over time. The sense of smell can be used to identify certain types of volatile organic compounds (VOCs). A strong yeasty smell may indicate that yeasts have overgrown the fermentation broth. Another category of sensory inspection is auditory inspections. When the grains are very active, one notices a fizzing sound.

To sum up, the culturing method is relatively simple, but challenging, and the value of utilizing the senses should not be underestimated or overlooked when monitoring the culturing process.

### **Data capture**

A Sony digital camera, Model DSC-W350 was used for image acquisition. Photographing and filming at up to 30 frames per seconds was performed during brief periods (<10 minutes) to record grain activity at random moments throughout the day. Images and/or video records were obtained by holding the camera very close to the surface and looking down on the culture, or from the sides through the clear glass while moving the camera around in search of a better view. In addition, short video surveys were taken to capture panoramic views. With this method, it was possible to track multiple grains simultaneously and extract relevant image frames. Background elements in the surroundings were valuable to give a sense of scale.

Daily changes to the grain network were documented. All records were totally dependent on the grains being observable. Grains can be viewed from looking over the culture or from the clear glass, without disturbing them. It should be noted that grains don't necessarily always float or face the camera. Because the grains are moving around and the medium is turbid, the visibility will change dramatically throughout the day and one can be prevented from seeing them. The precise time a grain disappears from view can be within a few seconds or minutes, giving a short window of time for observations to be made.

### **Data analyses and reporting**

Photographs and video records were analyzed retrospectively. Due to its dynamic nature, the ECM system generated large and unprecedented volumes of data. Such data typically are difficult to analyze and comprehend. All images were

retrospectively reviewed in a blinded fashion, and carefully examined under magnification (up to 30x). This was a laborious process entirely done manually. The work is not limited to the examples shown and described in this manuscript. Only the most illustrative examples are presented for clarity. Video recording are available as additional files.

### Morphological features

Biomass description included but was not limited to, branching network, crack openings, aspects of margins (smooth, irregular), pigmentation (color) and any other patterns of growth (which presumably reflect dynamic microbial population changes in nature). The relative pattern of shading and texture was examined to determine whether different parts of a grain surface are made of new biomass.

### Reference material (image collection)

A culture medium may contain hundreds of different unidentified grain particulates at any given time, making their identification more difficult. How is it possible to track the grain's transformation in a constantly changing environment?

A solid reference collection has been established showing the relative development sequence of select grains during successive cycles for the purpose of authentication. During the study that lasted four months, the grains have gone through many forms that are very different from their initial design. The observer has captured >1000 grain images. These images are searchable as the grains have distinctive features, although unfortunately the distinctiveness of these features is not so great as to make their identification a trivial task.

The image collection was analysed to establish the temporal link that connect one form to another and therefore determine that it is the same grain. No two grains are alike yet, they have structural similarities that could be used to see if there are related or not. Tracking was thought to be significantly easier for larger grains, but this was not necessarily the case. The analysis is complicated by the fact that many grains present complex aggregate structures in three dimensions (3D) that are constantly changing, so that real-time tracking is inevitably challenging. For simplicity, the grains are considered two-sided, with front and reverse sides; one or the other side may be facing the camera at any moment in time. With the current model, it is not possible to see both sides at once.

Most of the time, the images do not have the proper orientation. Serial comparisons between inverted images and all other orientations are needed to determine whether the grains are temporally linked structures based on morphological similarities. Each orientation in 45 degree-steps was analyzed for unequivocal identification of individual transformation sequences showing unique patterns. This approach is laborious, with many images being rotated and compared in different

orientation before a near-complete picture of transformation sequences begins to emerge. For select grains, the associated 3D forms have been laid out in chronological order beginning with the earlier form observed, and moving to the next forms, trying to select the most representative images available for presentation.

#### Collective motions and other behavior patterns

The grains were analyzed in their spatial and temporal context to identify behavior patterns in pairs and/or larger formations, looking at small changes in their position relative to the nearest neighbors that may reflect the possibility of interactions and/or close bonds. The grains are interacting with their surroundings, periodically ‘diving’ to explore deeper layers. It is impossible to predict the location of a grain at any given time.

The position of a select grain was examined relative to neighboring and/or more distant grains, and then checked for periodic shifts in position. The correct performance of this task requires shape discrimination and recognition of visual forms based on the relative development sequences established in the manner described in the above section.

## **RESULTS AND DISCUSSION**

### **Reconstruction results**

Starter powder cultures were investigated for their ability to regenerate KGs. This was part of an ambitious effort to produce a whole new generation of KGs through the use of a commercial starter that does not contain such grains. It was assumed that the active starter cultures sequestered in a milk pouch would have a better chance of forming KGs. After the last 24-hour incubation period, it was noted that the starter strains had undergone a dramatic transformation into pebble-like structures in their simplest forms, and others with coarse rounded and/or angular segments, varying anywhere from rice- to walnut-size (Fig 1). It seems that the grains were able to spontaneously reorder themselves. Most granules had an almost round or ovoid appearance, typically less than 1 mm wide. Others were noticeably larger (up to about 2 cm). The diverse and complex assemblages were visible as tan-colored masses, likely due to absorption of brown sugar. Similar results were obtained in triplicate.

Those grain particulates showed no visible signs of life until later, when placed in a whey-molasses solution at which time they exhibited clear signs of metabolic activity. Activation was inferred from the presence of CO<sub>2</sub> bubbles and extremely slow motions, barely perceptible with the naked eye showing that the KGs are living creatures.

The scientific evidence for reconstruction of KGs is finally there. To our knowledge, the present data are the first to show that it is possible to reconstruct KGs out of microbial isolates. This finding provides further support to the studies

which have hypothesized microbial surface auto-aggregation as an origin of these structures [10, 13]. A combination of events caused an amazing, unforgettable natural phenomenon to occur in just three days. The dramatic transformation was as remarkable as it was unexpected. All things considered, the KGs were given a second gift of life. It is assumed that they began their new existence with the formation of small clusters of bacteria and yeasts, and induction of capsular kefir by EPS-producing bacterial strains. It is likely that natural airborne bacteria and yeasts took over at some point to produce symbiotic communities closely interacting together, and able to survive and reproduce. The discovery became the starting point for further studies on KGs as described in the following sections.

### **Progression of milk culture**

The milk culture began by using the smaller grains (newly discovered) as initial inoculum. A typical batch of milk kefir is shown in Fig 2. The culturing process has generated an abundance of grains resembling cheese curds and/or cauliflower florets (Fig. 3). During the course of fermentation, the grains were found to be productive, transforming milk into a delicious beverage with a slightly tart and refreshing taste typical of authentic kefir. In the later stage of fermentation, i.e., after several days in refrigerated conditions, the aroma becomes stronger, typically yeasty; the flavor is refreshingly pungent with mixed lactic acid and other organic acids, and the texture is homogeneous, creamy, and effervescent, in accordance with the description of fully-fermented kefir given by [4].

The results demonstrate that the grains have blossomed into a reliable mother-starter and have been reproducing under continuous conditions for over three years. Making sure that they are fed daily helps ensure the milk kefir produced has the best flavor profile.

### Microbial composition

No attempt has been made to analyze the microbial composition by cultivating the strains under laboratory conditions. Instead, the reader is referred to the published literature. A detailed description of the typical strains most commonly found in traditional kefir and the grains themselves is given in references [8-15]. Many species existing as a consortium in the grains would have been difficult to isolate and culture separately.

The functioning of the community depends on a delicate balance between different strains of bacteria and yeasts [5]. The yeasts have a narrower pH and temperature growth range, so they are more difficult to maintain than bacteria. Aeration to stimulate yeasts can be achieved through vigorous stirring of the milk prior to adding the grains. The dissolved oxygen will be used by the yeasts to form new cell membranes. In the presence of oxygen (i.e., under aerobic conditions), the yeasts

can grow to higher density and consume sugars that are completely metabolized to CO<sub>2</sub>, energy and water. Under anaerobic conditions, the yeasts grow more slowly and sugars will be, only partially, metabolized to ethanol and some CO<sub>2</sub>. They produce growth factors in the form of amino-acids and vitamins. They consume lactic acid produced by bacteria, trying to keep the acidity low while EPS-producing bacteria consume alcohol produced by the yeasts.

Based on its sensory characteristics, it can be speculated with high confidence that the kefir produced in this study contains aromatic mesophilic cultures, moderate acidifiers with some CO<sub>2</sub> gas production and creamy/buttery flavor due to high EPS production and diacetyl production.

### Importance of proper maintenance

The culture must not be left neglected. Continuous propagation of the grains in culture at intervals of 24 to 36 h is necessary to keep the correct balance of microorganisms. A life out of balance usually means the culture will not be producing productive grains. The grains are designed to grow into maturity. They are no more than the size of a coarse sand particle in early stages; some increase in size and porosity occurs within each cycle. The grains become more complex and clusters begin to be more evident over time; the larger surface-to-volume ratio seems to better control the flow of nutrients and cellular materials into and out. Some grains may look structurally 'distressed' and may never reach maturity. When KG growth is disturbed, they deteriorate and lose their resilience; they shrink in size, some disappear and become indistinguishable.

It is essential for KGs to maintain their ability to self-propagate through repetitive (daily) fermentation cycles. They contain naturally occurring bacteria and yeasts that have the capacity to survive indefinitely when maintained in this particular habitat. The organic acids (lactic acids, acetic acid and fruity esters) and odorous alcohols provide a pleasant taste and smell, and also inhibit the development of undesirable or pathogenic microorganisms, in association with other naturally-produced preservatives [7]. There may exist in milk kefir, under normal circumstances a small amount of beneficial molds (*Penicillium Camemberti* and *Geotricum Candidum*). These rapid growing molds prevent unwanted mold growth [4].

The culture is subject to natural environmental factors that are not easily controllable. A single grain can harbor millions of cells living in harmony. There is evidence that balance may be disturbed by various forms of stress. Certainly, some of the variability is due to varying culturing conditions primarily due to seasonal variations, and a very low number of grains likely represent grains not kept under ideal conditions. Since any protocol deviation may reduce the frequency and number of KGs produced per batch, a recovery protocol was tested and found to be useful to restore the integrity of grain cultures, as needed.

Results from three recovery experiments were consistent. There is direct experimental evidence that the grains could spontaneously reorder themselves under certain circumstances. Macroscopic appearance of the newly emerging grains is depicted in Fig. 3. Their productivity demonstrates that the balance of yeasts vs. bacteria has been regained. These findings corroborate published observations indicating that supplementation with different minerals and vitamins may promote the growth of KGs [41].

### General recommendations

Both detecting and understanding the visual signs of a slow culture constitute the first step to overcoming potential problems that may occur during the course of the fermentation process. Culturing the grains for over three years has provided the author a way of gaining more knowledge about what an ideal maintenance protocol might be through direct observations and hands-on experience. The detailed description of the culturing process provided in this report should enable everyone who is interested in KGs to see what is necessary to get started. It is important to always adjust the amount of milk relative to the grain mass so that the grains are not too diluted or concentrated. The porous interior and irregular surfaces of grains provide ideal conditions for cell growth. When all conditions are favorable, good results may be obtained.

Beginners are strongly advised to become familiar with the culturing process to keep the culture in good condition. There are some common signs of inefficient fermentation. A few examples are listed below. By recognizing these early signs, one can adjust the culture conditions in such a way as to promote growth of KGs and get the highest possible quantity and quality of KGs. The improved sensory characteristics of the kefir produced will be worth the efforts.

- The grains have entered dormancy and do not multiply, which does not necessarily mean that they are not productive. It is recommended to increase fermentation time from 24 to 36 h, and insulate the culture from cool air.
- The grains are scarcely visible and look structurally distressed. They lack the complex surface clusters and porous structures that are distinctive characteristics of well-formed grains. Vigorous bubbling action is seen after agitation. It is possible that too much acidity produced may have hindered the growth of yeasts inside the grains. Nutrients may have become exhausted due to high cell density and warmer temperature.
- Grains are well-formed suggesting capsular EPS production has occurred, but the grains have poor quality. The kefir produced is watery and bubbly. It is possible that a dominant yeast strain took over the fermentation. The best way to mitigate this problem is to reduce yeast activity and reinforce the bacterial components.

Over the years, the author has found even more ways to further improve culture performance which relies upon the delicate balance of bacteria and yeast communities. Creating a stable microclimate has proven to be useful to help maintain the grain cultures. It is clear that the presence of oxygen, as well as optimum air temperature and moisture are needed to stimulate the wild variety of bacterial and yeasts strains. During respiration, the yeast produces CO<sub>2</sub>, moisture and heat. A large plastic container with sufficient depth serves as a perfect microclimate chamber to keep air temperature and moisture more constant, and also trap volatile organic compounds that seem to benefit the cultures.

To sum up, kefir production is a process that involves microbiological and physicochemical changes that affects the culture in many different ways. Sensory properties of authentic kefir come essentially from the grains themselves and how they are grown. To preserve a reliable culture, the importance of routine monitoring cannot be overstated. When the grains have almost reached a point of no return despite the greatest care, it is still possible to stimulate their growth. Kefir should be fermented continually in the presence of KGs, and long enough to give its best flavor and texture; it is our opinion that prolonged chilling produce the strongest and most desirable kefir.

### **Experimental culture model system**

An ECM system was developed to study the larger grains (N=12, newly discovered). The grains were cohesive enough to be recovered and studied through repetitive fermentation cycles. In this system, they differentiated into more complex visual forms. They can move in any directions and turn over by themselves, looking different. All these situations have made it difficult to track them in a systematic way. The absence of a particular grain in the field of view does not mean that it is not present in that environment. Despite the numerous challenges, it has been possible to make sense of the growing volumes of observational data collected over time and convert them into meaningful information. This experiment model provided a window into a hidden world as never seen before. The key findings are summarized below.

### **Dynamic transformation**

During the first weeks, most of the aged grains grew relatively slowly, but then suddenly developed on subsequent weeks, with increasing complexity, once fresh grains had been introduced into the medium. The image collection database captures the temporal link that connect one form to another. The grains have different morphological features allowing them to be differentiated from one another (Figs. 4 and 5). Note the changing surface patterns through successive fermentation cycles. The branching network and hollow structures are more prominent as the grains differentiate into more complex visual forms. The grains have undergone a radical transformation: although they have the same bodies, they are not all the



same as they used to be. It is not known which microbial strains are responsible for these changes; the porous structures and internal cavities however, attest to the need of KGs for growth nutrients and cellular materials circulating through the biomass.

### Collective motions

There is no doubt that the KGs seem to have a good sense of their surroundings. They have natural curiosity. When fresh grains are introduced, the aged grains are more likely to pick at them. Supplementation with fresh grains provides a continuum of microbial life forms beneficial to the entire community by forming many new boundaries throughout the culture. Together, the grains become part of a larger community and create their own slightly warmer microclimate.

Their motions are diverse and difficult to predict. They are sensitive to the slightest turbulence created by CO<sub>2</sub> bubbles. Occasionally, the grains initiate some movements that are captivating to watch as exemplified in Fig 6. Some grains are more mobile than others, and seem to regularly patrol their complex surroundings in search of nutrients. Close observations show that grains can shift in different directions, presumably depending on a number of invisible stimuli. They often float at the water's surface gathered in groups or reach deeper layers before resurfacing, presumably seeking new synergistic relationships. Groups of grains move slowly near the surface throughout the day; their extremely slow motions are barely perceptible with the naked eye, but it is clear that they do not limit the area of searching to the surface. Occasionally, they are seen speeding up their movements, twisting or turning over by themselves before diving to reach deeper layers and then come back up. During searching activities, they seem to have precise knowledge about the spatial location of resources and attempt to cover the maximum area possible.

This local searching behavior presumably reflects the complex dynamic microbial population changes in nature, influenced by temporal changes in temperature, nutrient gradients and viscosity due to EPS production – known to be stimulated by the addition of *S. Cerevisiae* [42].

### Self-propagation

Kefir grains are known to propagate by fragmentation in milk. The clusters liberated from the irregular surface ('baby-grains') turn into mother-grains [18]. Finding visual examples of self-propagation and baby-grains in the ECM system was expected to be difficult; this was a far from trivial undertaking in view of the complexity of the culture: remember that a culture may contain hundreds of different grain particulates at any given time. Figs. 7–9 summarize some of the key events that have occurred during the life cycle of two KGs from their early days through maturation period. Various visual forms

are rendered through a combination of shearing, thinning and surface covering by new biomass visible on the exterior surface. With each advance of the cracks and splits, the fragmented structures are repaired with new building materials filling the gaps. All these results confirm that the grains undergo fragmentation as part of their natural cycle; they are unstable but they are resilient and continue to exist. All ecosystems provide a limit in accumulation of biomass. After being partially degraded, a grain will often sink, at which point decomposition and/or rebuilding may begin. As time passes, clusters of biomass are continually released through cracks and splits, leaving remnant structures that are being revitalized to be carried on to the next generations.

### Behavior patterns

In contrast with the traditional method of growing grains in milk, the ECM system provided colorful scenery with a diversity of moving forms that grabbed the viewer's attention. By looking attentively, it became possible to characterize the culture in terms of how the grains relate to one another as a close community. It is absolutely clear that the grains live in harmony in an ever-changing environment around them. The aged grains appear to continually explore the landscape around them and engage in some form of communication with neighboring grains. Fresh dairy grains do not differ much in their behavior from the aged grains even. They also seem to actively seek new relationships (Fig. 10). The data suggest that grains can detect the proximity of other grains and derive sufficient information to orient in a directional manner. They seem to have precise information about the proximity of other grains and spatial location of resources. This local searching behavior gives them an opportunity to interact with many others in the larger community as they attempt to cover the maximum possible area. This is indeed a very transformative world, subject to resources demand due to the diversity of microbial life forms involved in the community.

In this system, the grains remained diverse and productive, imparting a deep golden color, flavor and aroma to the medium. Monitoring the behavior of the grains contributed to the successful outcome of these studies. Knowing where and how the grains are spending their time could aid in evaluating whether the culturing conditions are effective. During the first week of culture, this was a particular challenging habitat where select grains (N=12) were hardly able to grow singly cultivated. Those grains were considered the best material for studies on KGs but soon it was realized that the cultures needed environmental enrichment to encourage a wide range of natural behaviors and keep their life interesting and challenging. In this ecological niche, the fresh grains added later, played an important role by contributing to the formation of a larger community, causing the grains to display all kinds of natural behaviors ranging from exploration to close bonds as

never seen before. As yet, the use of this system must be deemed experimental only as it is not an ordinary way of cultivating KGs, and must not be continued for too long as the tendency may be produce an imbalance over time.

Watching those grains periodically made it possible to capture some of the most intimate moments of their lives as they come close and interact with each other. Tracking was difficult among visually similar grains, yet possible because most grains were distinct in a combination of features, i.e., the conjunction of color, form and pattern that made them visually unique, and easier to track (Fig. 11). Behavioral data collected could tell how much time each grain spends on the surface or at the bottom, as well as how they interact with each other and their environment. The grains have individual ‘personalities’ and preferences. During the search for certain behavior patterns, the author has witnessed unique and captivating views of the grains going under water, then coming back up, mutually engaged in some sort of ‘courtship’ behavior. Remarkably, direct experimental evidence of pair formation was found no matter how crowded the culture had become. The grains appear to be engaged in some form of communication and there is a sense of intimate connection taking place (Figs 12 and 13). A few times, they are so near each other that their surfaces almost touch.

The data suggest that some grains have the remarkable ability to reconnect with their original partner(s). This curious pattern of behavior was extremely difficult to observe due to the random motions of grains. It is intriguing to think that some grains are interconnected both in space and time, and seek to be reunited periodically. The grains have been structurally altered but close bonds have remained. As strange as it sounds, it was possible to use information from one grain to locate the other grain(s) moving in the vicinity (refer to additional video file).

Although many gaps remain in our fundamental understanding of this phenomenon, this is definitely not a random association that can simply be explained by probability events. The probability that pair bonding is due to a chance alignment is small. These pairing interactions were too frequent to be a coincidence. Also, the fact that the pairing was observed with grains in motion and even in crowded places strengthen the earlier observations. The author used the most accurate way to record observations through photographs and videos, thereby giving most compelling evidence that this may be a real phenomenon.

Based on the above scientific observations, there is no doubt that KGs constitute one of the most intriguing and diverse ecosystem with a display of close relationships. This is a different medium from milk, and in this ecological niche, only well adapted microorganisms are able to grow. Why do the same KGs are periodically attracted towards each other and appear to return to the same partner from the past, no matter how crowded the culture has become, is not known. It is not within the scope of the report to discuss in details, the intricacies of these symbiotic relationships. It is plausible that intimate connections occur through transmission of chemically complex molecules from one partner to the other, and/or signaling-

based communication. Excellent reviews of metabolic activity and symbiotic interaction of bacteria and yeasts can be found in reference [43-44].

It is well known that bacteria in particular, can follow gradients of nutrients and other environmental stimuli. Interactions may occur via chemotaxis (directed motion) and cell contact. Cell-to-cell communication is a complex phenomenon which cannot be simply described, and extensive research has been undergoing. Bacteria cells that have aggregated can communicate information about population density and metabolic state (called quorum sensing) and release special signaling chemicals [45-48]. Regardless of the mechanism involved, each grain provides a continuum of microbial life forms able to survive as a close community by gaining support from each other. Survival depends on the many contributions of each participating grain within the culture. The presence of fresh grains together with aged grains facilitated a kind of 'social' network. Together, the grains build strength through symbiotic alliances which help the microbial community grow, adapt and survive together over multiple generations.

## **CONCLUSIONS**

The present study is the first one to provide scientific evidence that KGs can come back into existence and recover after being 'disembodied'. Just because select strains are separated from original grains and packaged in a freeze-dried sealed packet, it does not mean that the grains somehow have ceased to exist. The author was captivated by the intricate complexity of the newly-formed grains, each one having a unique design. The ECM system opened a window into their hidden world as never seen before. It could be seen that the grains are living creatures that share a special connection as they congregate in pairs and larger groups. As part of a close community, they move in different directions and interact in unforeseen ways which made them interesting to watch.

The natural starter developed in this work is considered unique because of the exceptional circumstances that led to its discovery. The grains were given a second gift of life; they are there to stay for decades to come as long as the cultures are given good care and the closest attention. The author invites the readers to experiment on KGs. The journey of discovery with these amazing creatures never ends.

## **FUTURE RESEARCH DIRECTIONS**

There is still a lot more that one can possibly learn about these grains. The ECM system has permitted an unprecedented, real-time view of pair-bonding behaviors. At present we do not fully understand the nature of these interactions. More studies may be useful to understand how the grains relate to their surroundings, and determine the way

morphological changes can influence their interactive behavior(s). This work raises many other interesting questions about how the grains communicate with each other, and can transform into sustainable life forms. In this regard, it may be interesting to characterize the diverse strains involved in this complex ecosystem.

## **ACKNOWLEDGMENTS**

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## **CONFLICT OF INTEREST STATEMENT**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The author does not have any financial interest in or arrangements with any company whose product was used in this study.

## **ELECTRONIC SUPPLEMENTAL MATERIAL**

Additional video file (complementary to Figure 12): Evidence of pair-bonding showing grain C naturally brought back to the surface and moving towards grain F. The grains collide and interact as a pair seemingly affecting each other. Note the CO<sub>2</sub> released in discrete bursts. MP4 689 MB, 29 frames/second.

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**Fig 1. Results of reconstruction experiment showing the dramatic transformation of starter powder cultures into a whole new generation of kefir grains.** (A) Shown from left to right are the experimental set up with milk pouch containing starter cultures (arrow) and the newly discovered grains collected in a fine-mesh strainer and suspended in fermented whey. (B) Shown from left to right are a plain view of the distinctively larger grains (N=12) in whey-molasses solution alone and then nestled among fresh supplemental grains (arrows). The aged grains are between 0.5 and 2 cm. The last image shows mixed grains collected into a pile and further examined prior to the next cycle; notice the diverse and complex assemblages; all grains have turned deeper brown due to absorption of molasses. Time-series images at intervals ranging from days to weeks.

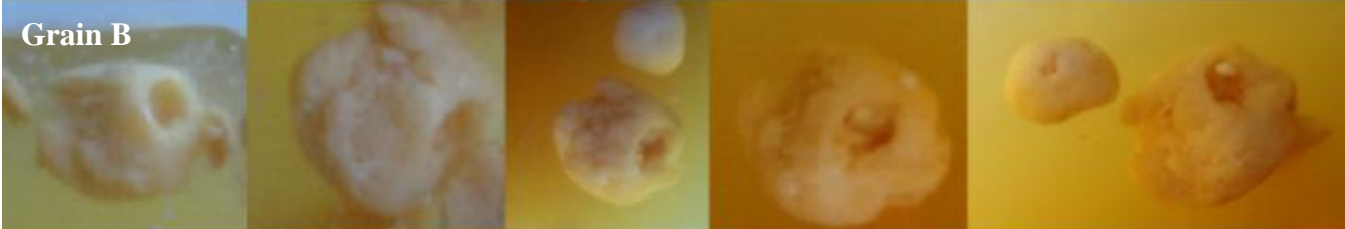




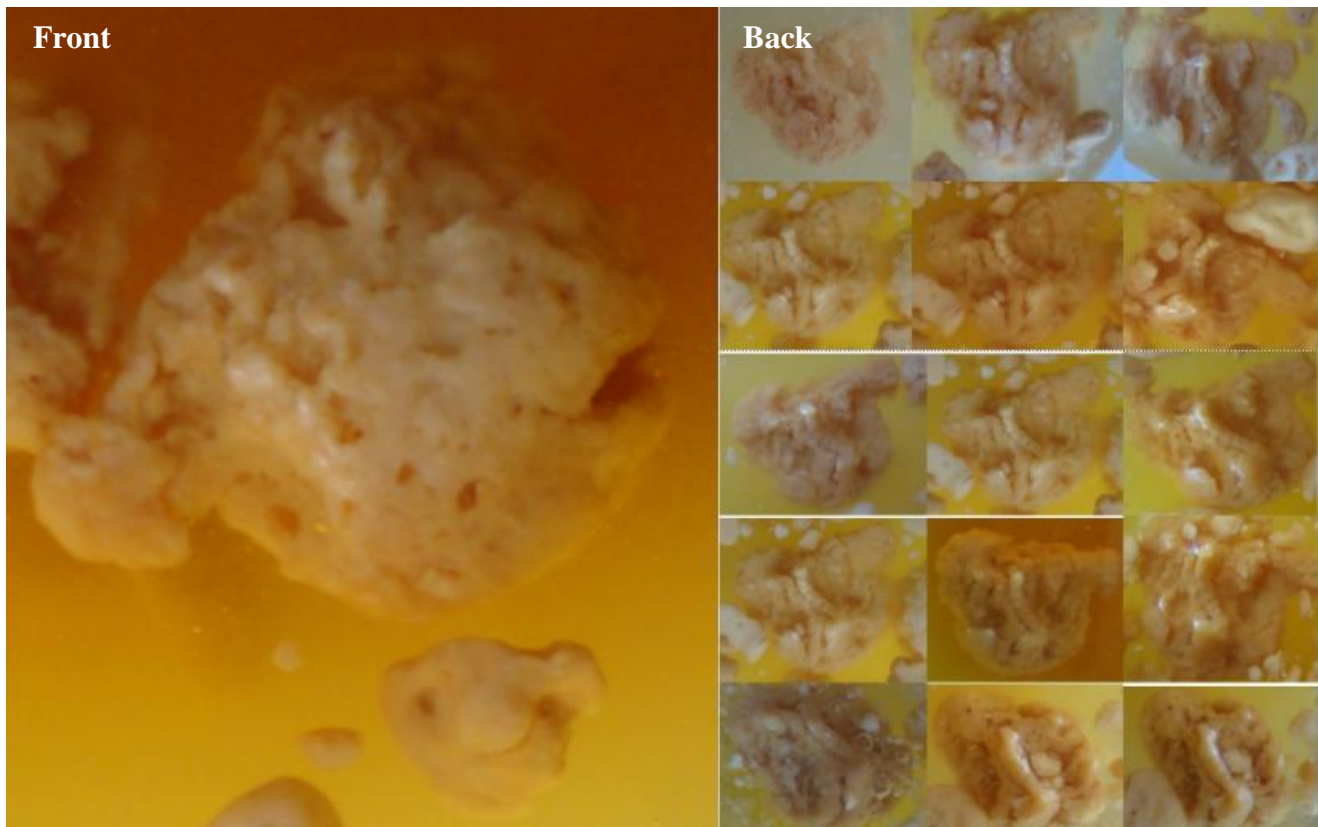
**Fig 2. Typical batch of milk kefir.** After a first fermentation cycle, three distinctive layers are visible. '1' = fermented whey liquid; '2' = smooth gelatinous layer with strands of kefiran-like substance; '3' = large number of KGs embedded in thick curds. Close up views show the expanding network of grains conglomerating on the surface as well as in whey pockets visible through the clear glass. The CO<sub>2</sub> bubbles burst over time forming 'eyes'. Notice the complex geometry and porous structures with lacy patterns. Some grains resemble cheese curds and/or cauliflower florets. Look closely and you will find that they are different.



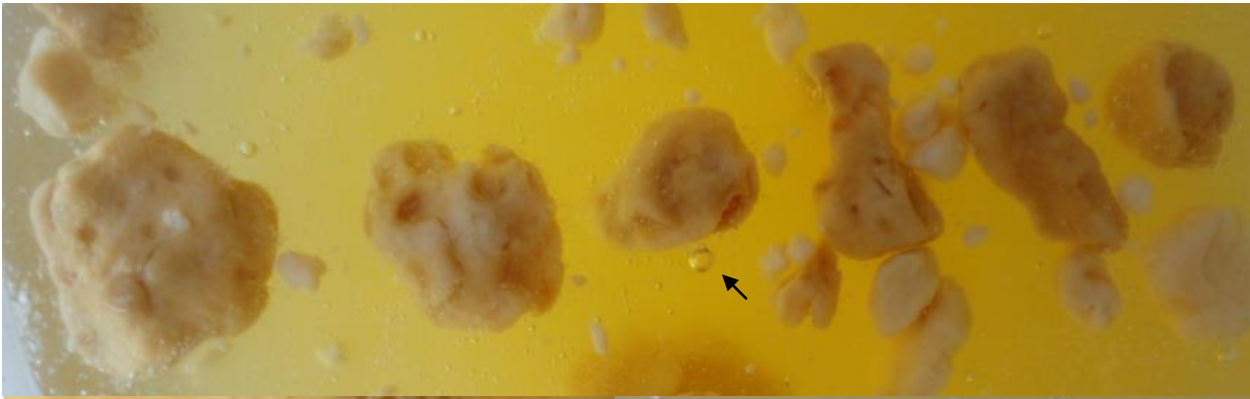
**Fig 3. Morphological appearance of newly-emerged grains (see recovery procedure).** (A) The culture is showing signs of recovery: a very thick, foamy layer has risen to the surface with KGs half-hidden inside. (B) The newly-emerged grains are golden brown and have interesting looking geometric shapes. (C) After passage in milk, three distinctive layers are visible similar to Figure 2. (D, E) The grains have retrieved their typical appearance, showing closely packed clusters of new biomass. No two grains are the same. Note small clusters sometimes referred to as 'baby-grains' (about 5 mm) branching off the main bodies, not yet liberated from the mother-grains. The largest grain weighs 2 grams. Total weight is 8 grams.



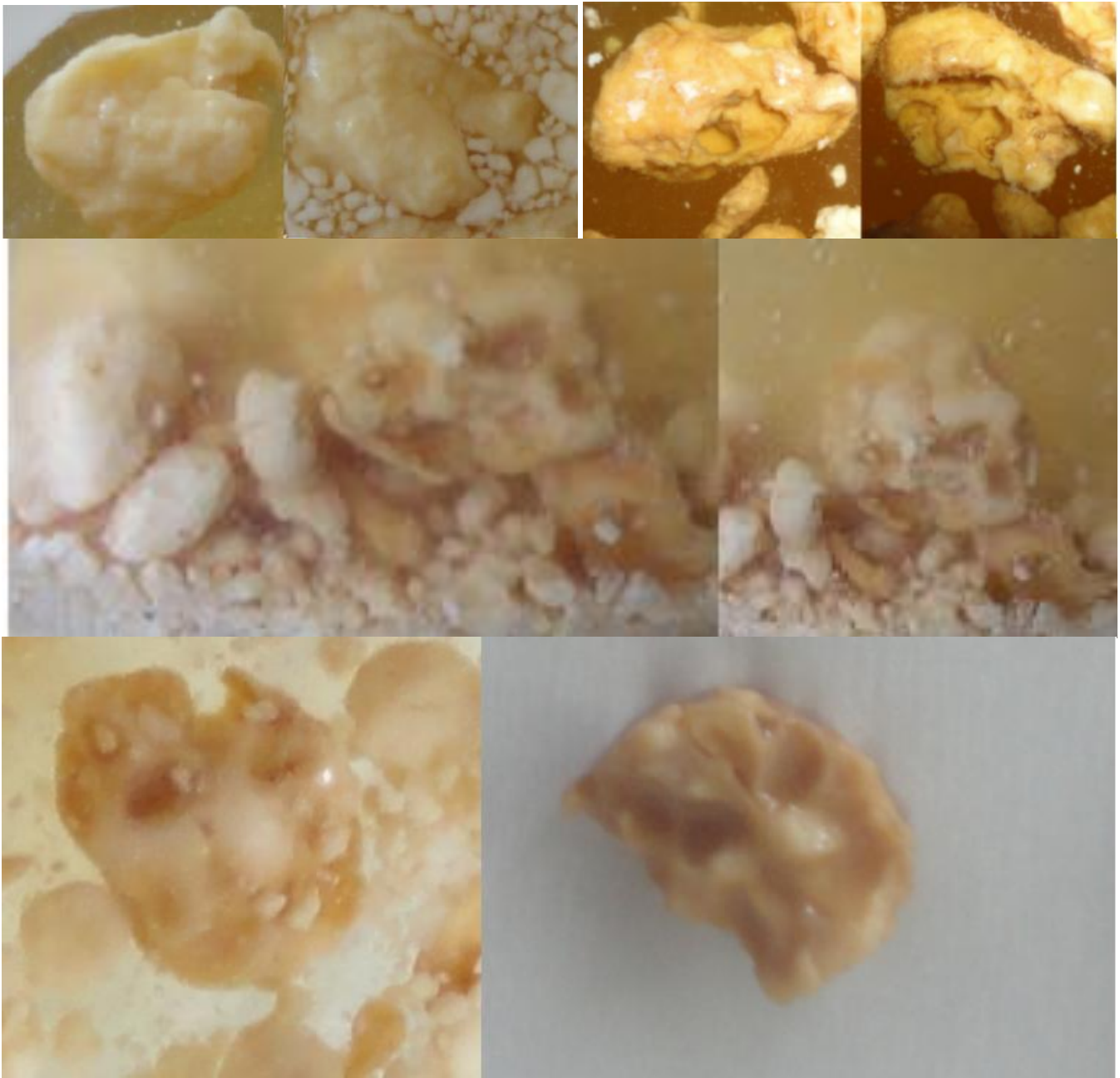
**Fig 4. Reference material from image collection, showing the relative development sequence of select grains established during successive fermentation cycles for the purpose of authentication.** The grains are labeled A, B (fresh grains) and C through G (aged grains) to facilitate tracking. Most grains have gone through many changes since the time they were first discovered. When held in the ECM system, some grains have developed more rapidly on subsequent weeks with increasingly complex forms. Grain G has become larger due to coalescence of neighboring grains. The starting grains are ~1.5 cm, based on mesh size. Time-series images at intervals ranging from days to weeks.



**Fig 5. Complex visual form of a fresh grain in whey-molasses solution (grain labeled H).** Close examination shows intricate interlaced surface patterns on either side. The grain floats so one side is exposed ('front') and the other side is submerged ('back') but visible after the grain has turned over on its own. The smaller grain in the vicinity (~0.7 cm) (see Figure 9) gives some sense of scale. Time-series images over a few days.



**Fig 6. Grain movements were captivating to watch (grain labeled F, see Figure 4).** In the first image, the CO<sub>2</sub> gas is enclosed within the grains so that they float. The most active grain (marked by arrow) is seen perusing its surroundings, reaching deeper layers and resurfacing. Notice the stream of CO<sub>2</sub> bubbles released from the inner structures. The grain discharges the excess CO<sub>2</sub> gas while regaining buoyancy. There is enough CO<sub>2</sub> being produced and trapped inside to lift it to the surface naturally. The grain has tilted in a different direction; changing orientation and repositioning causes it to display different visual forms.

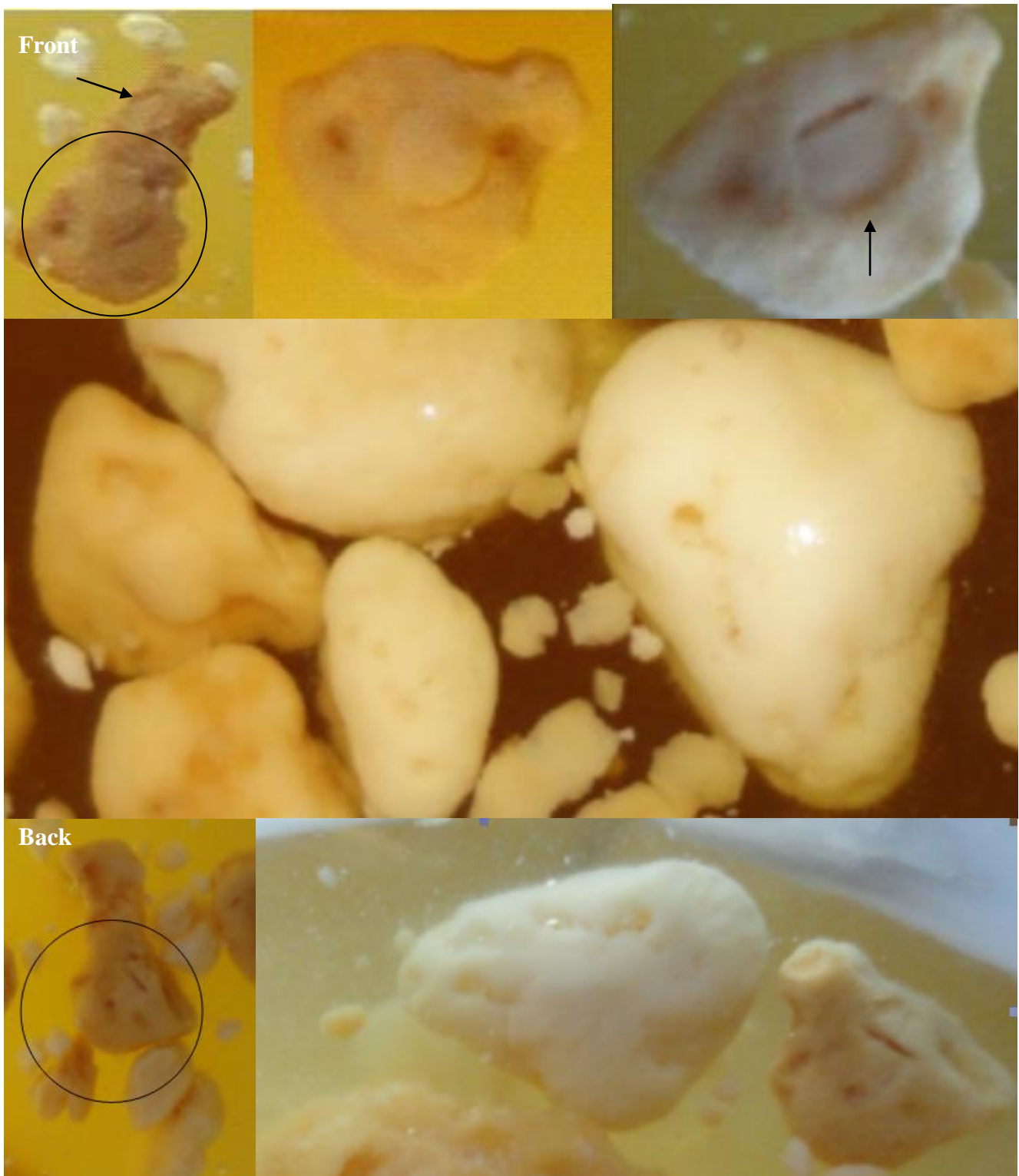


**Fig 7. Illustrative example of self-propagation (grain labeled E, see Figure 4).** This particular grain has gone through a succession of different situations with time, and this corresponds with changes in its original structure. After apparent weakening and partial breakdown, the grain has moved to deeper layers – the presence of grain debris and EPS secretions (visible with the naked eye) – makes the bottom layers a favorable habitat for colonization of fractured grain surfaces. There is enough CO<sub>2</sub> being produced by the new biomass to lift the grain to the surface naturally. Time-series images over three weeks. Starting grain size is ~2 cm. (see Figure 8; the same phenomenon has been observed on the opposite side).

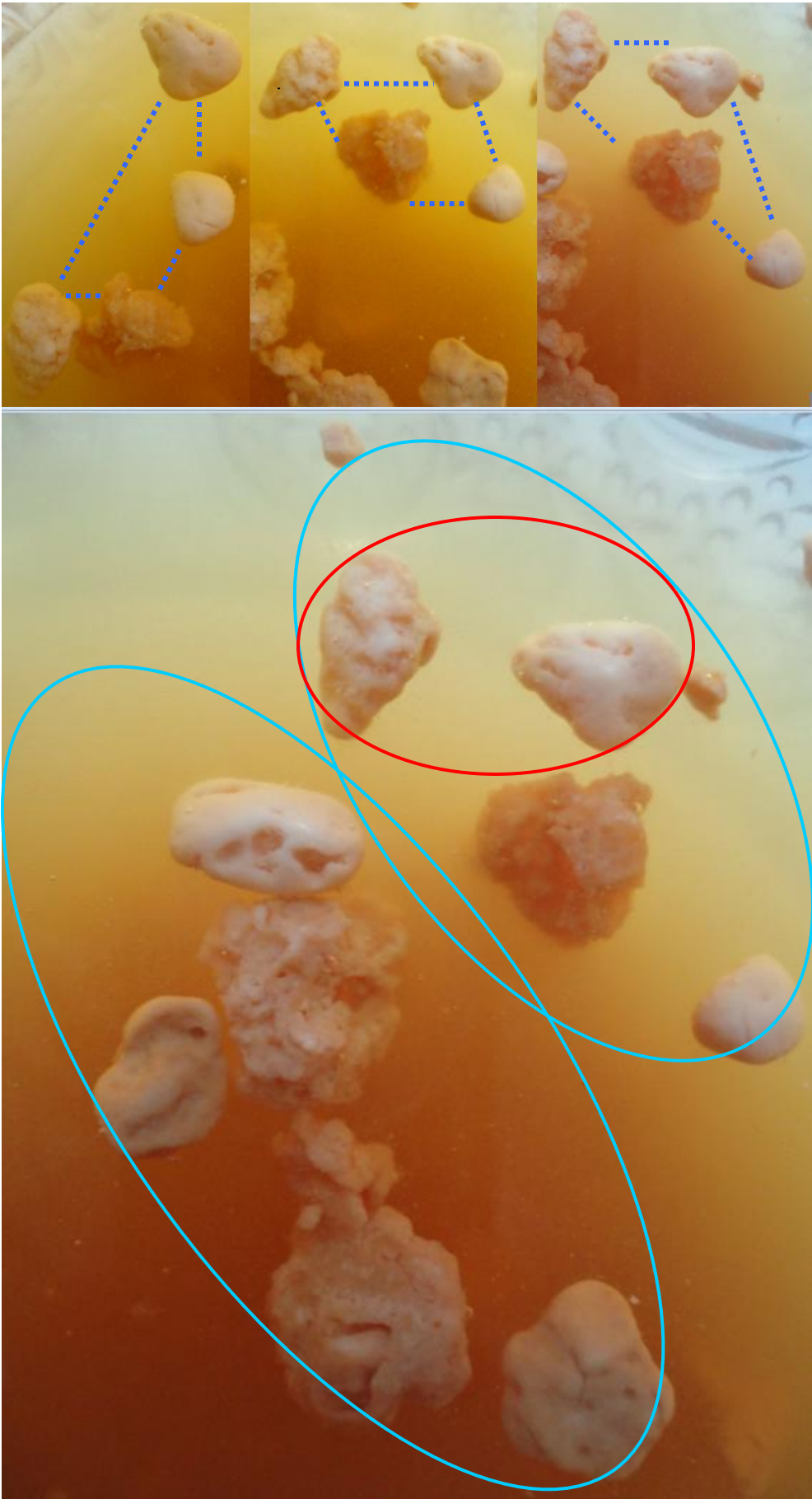


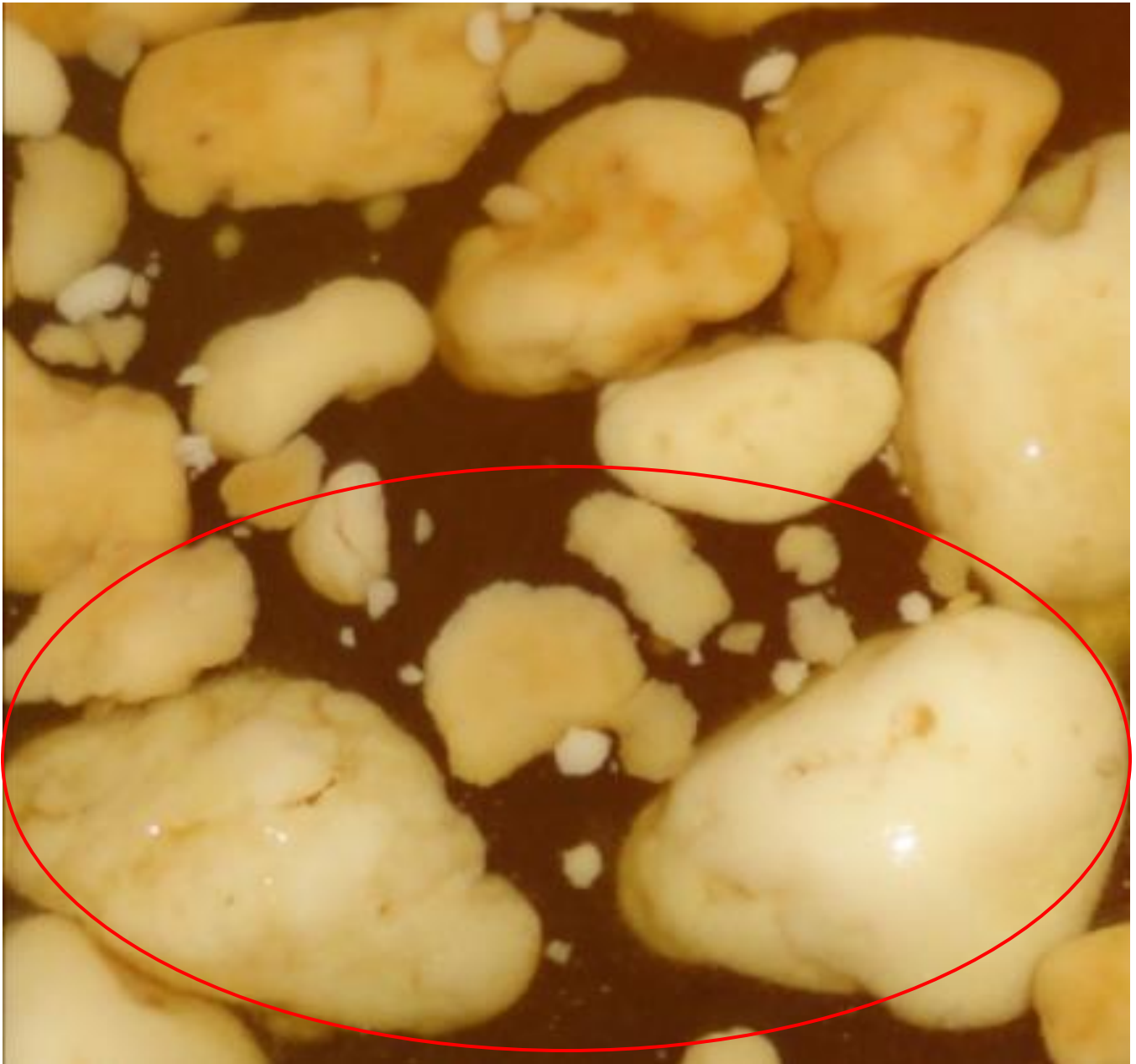


**Fig 8. Confirmatory evidence of self-propagation into a ‘new grain’ (grain labeled E).** Similar clues of revitalization were found when the grain has turned over, thereby exposing the other side. The fresh grains have a stimulatory effect due to their large mass and numbers. Time-series images over three weeks – the photographs in fourth and fifth positions are identical, except for an orientation difference. Notice the similar contours (compare with Figure 7).

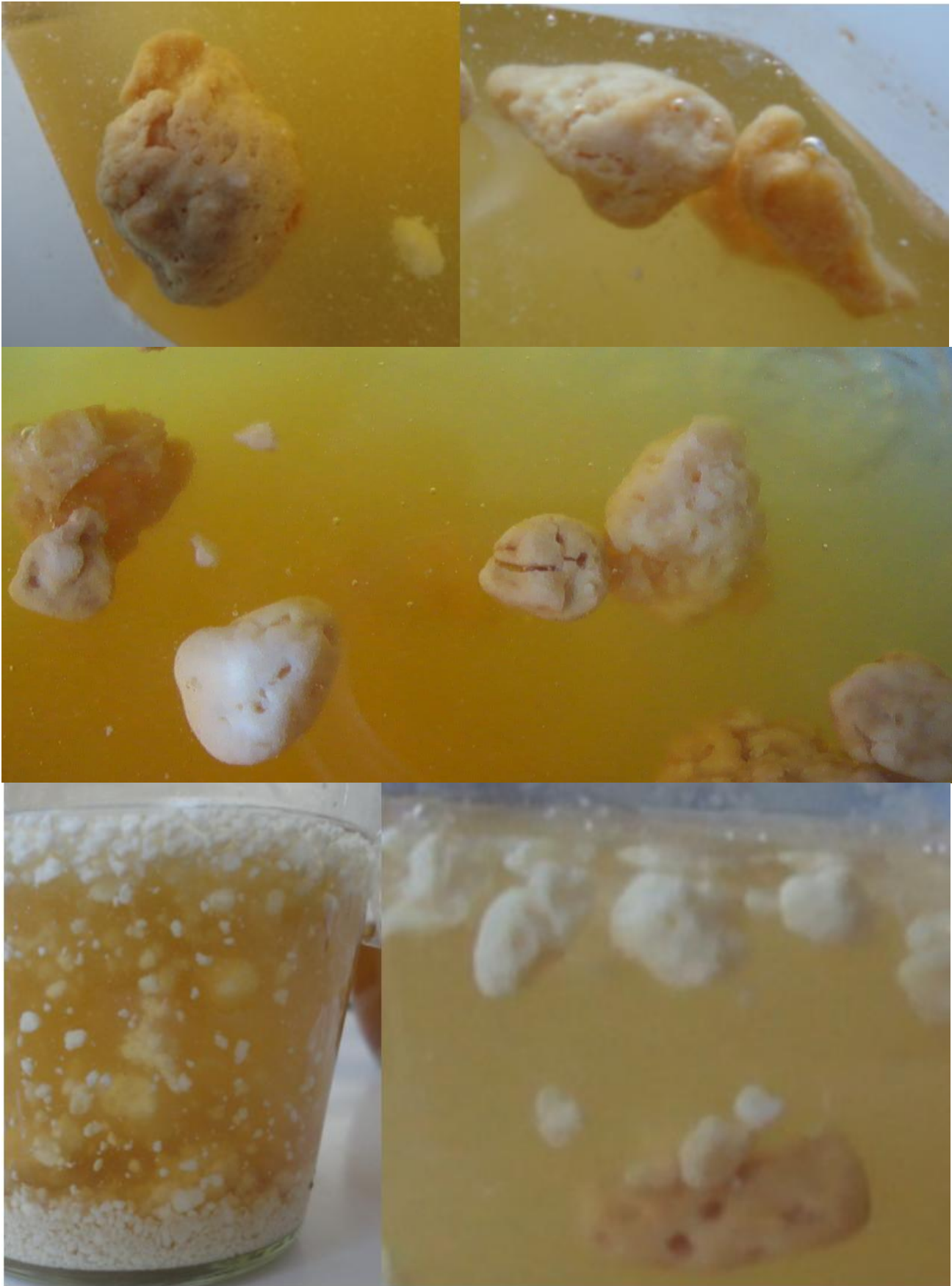


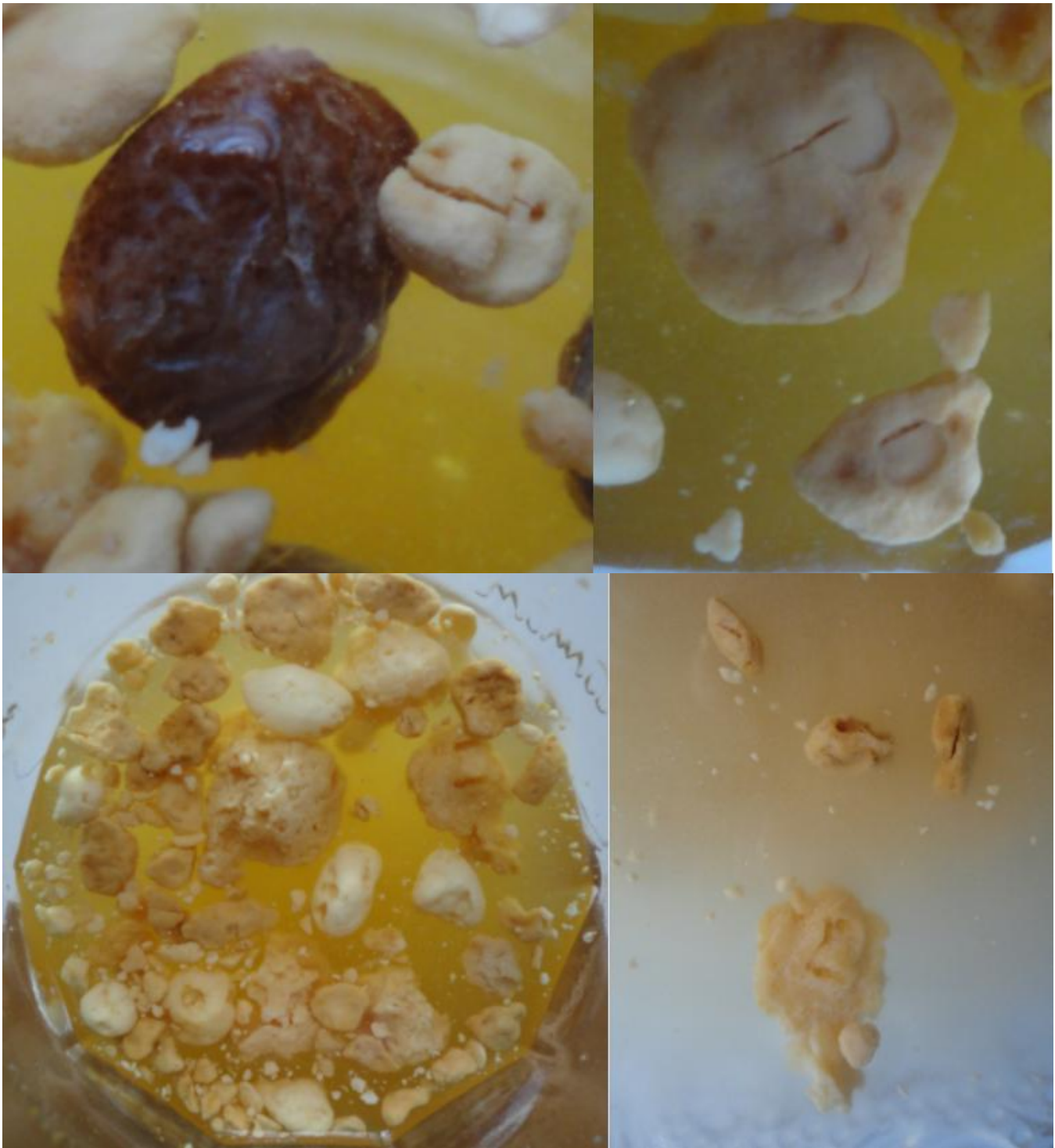
**Fig 9. Another illustrative example of grain self-propagation (grain labeled I).** This is one of the best documented examples of sequential crack growth and splitting events. Since a baby-grain was found embedded in the mother-grain (circled in black), one would expect to see it on its opposite side. Because the grain has turned over, the other side has been exposed (compare front and back). The same 'close tie' is seen on the opposite side. Note the bud formation at the zone of splitting. The pockmarks (arrows) suggest continuous release of globular clusters (assumed to represent nascent grains, ~2 to 3 mm). The starting grain is ~1 cm. The baby-grain is ~0.7 cm. Time-series images over three weeks.



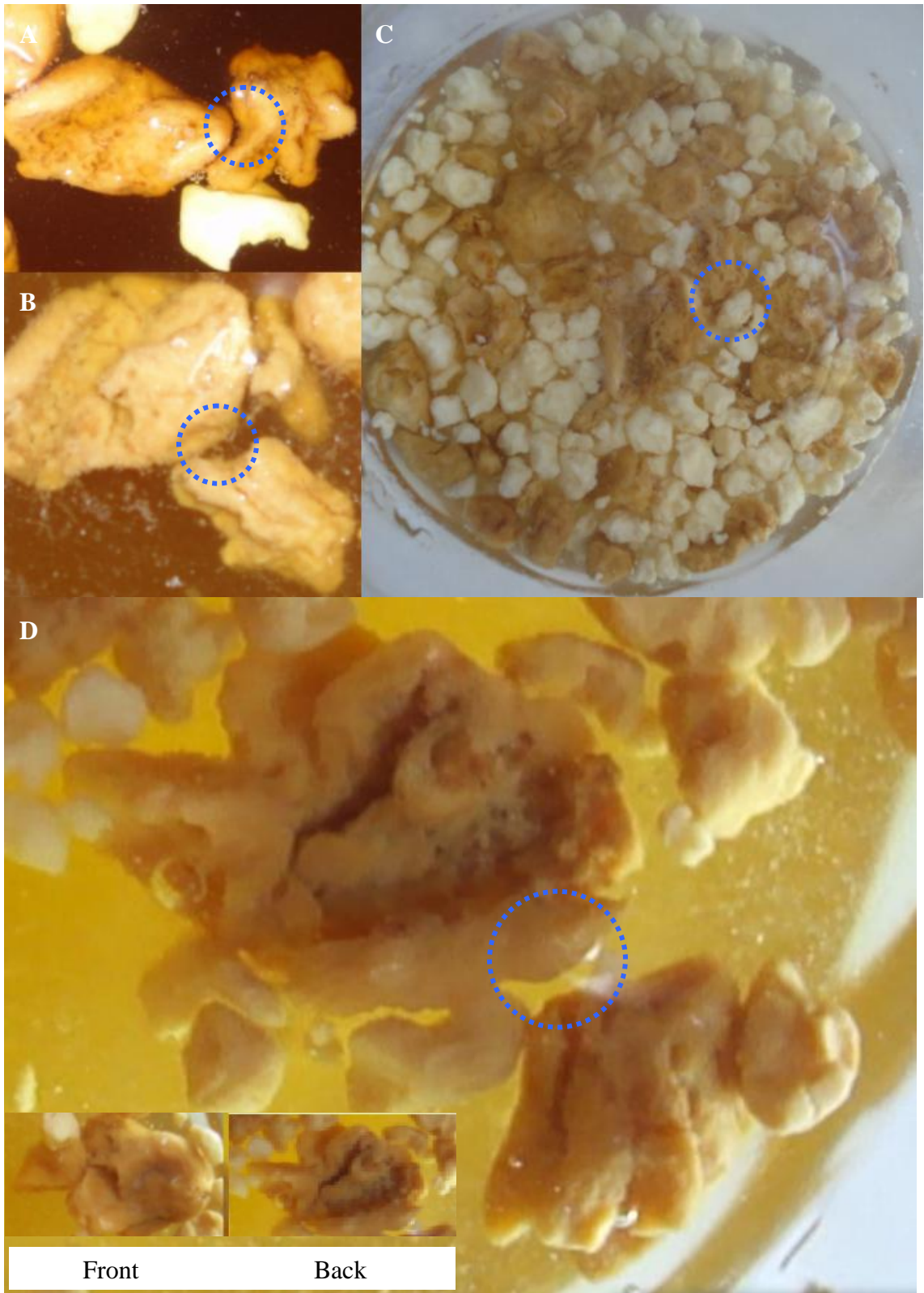


**Fig 10. Special grouping and motions captured in a series of panoramic views.** When comparing the three-panel composite image, it can be seen that the relative orientation of any given grain with respect to each other is quite different. Within one minute, the grains have gotten closer to each other, changing their position in response to invisible stimuli (dotted lines). There is a sense of intimate connection accentuated by their physical proximity. Within each group (circled), the participating members are seemingly engaged in 'cross-talk'. In the last image, one can see a pair of grains (red circle) in close proximity (the same pair is visible in the other images). Grain sizes between 0.5 and 1.5 cm.

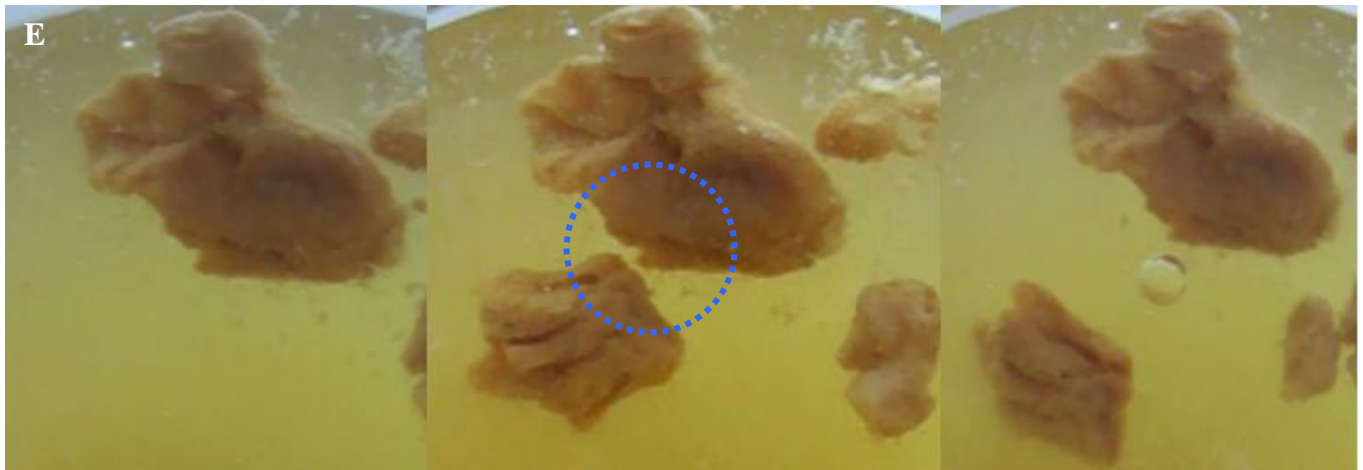




**Fig 11. The grains are living in a close community.** The ECM system brought a variety of grains together. The grains are fascinatingly complex mixtures of living microorganisms that receive a significant advantage from interacting with each other, and various biofilm surfaces, the most important being to share resources, adapt and survive. Together, they continually explore the changing landscape around them, presumably in search of symbiotic relationships. They seem to be attracted by the yeast-rich surfaces. Plumped fruit (sun-dried date) has risen to the surface during active fermentation and floats next to an aged grain. Images were obtained on different days, when looking from over the culture and/or through the glass walls.



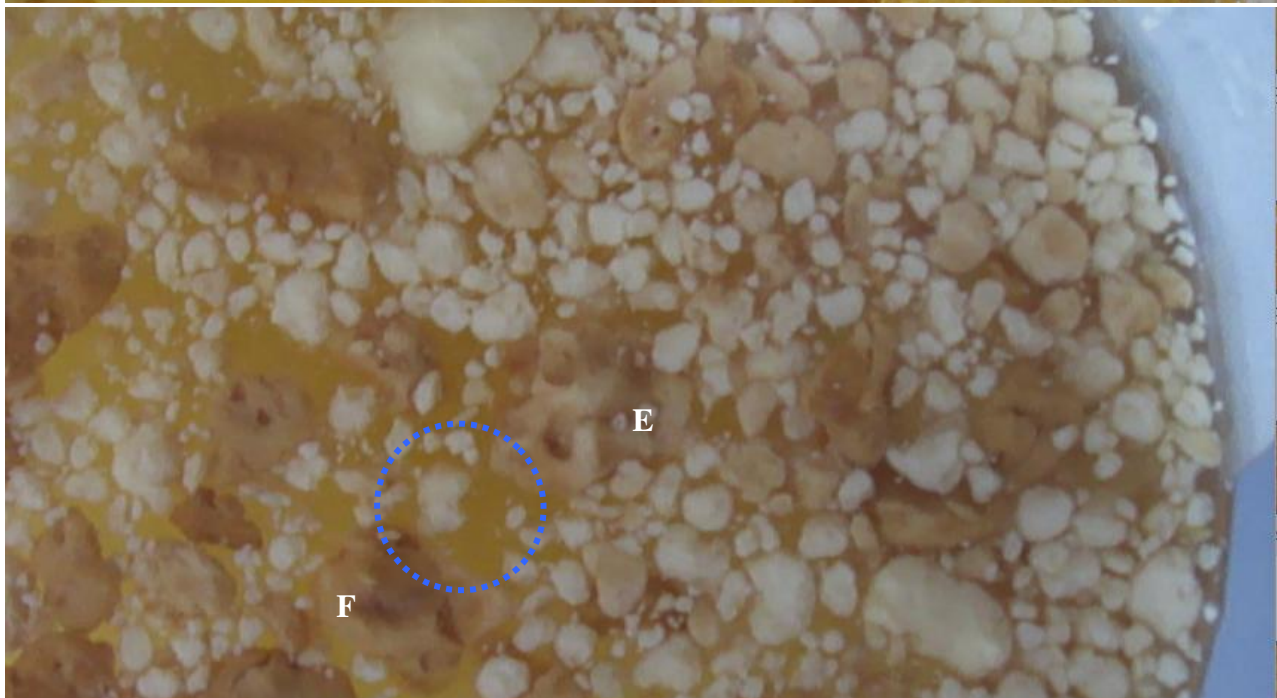
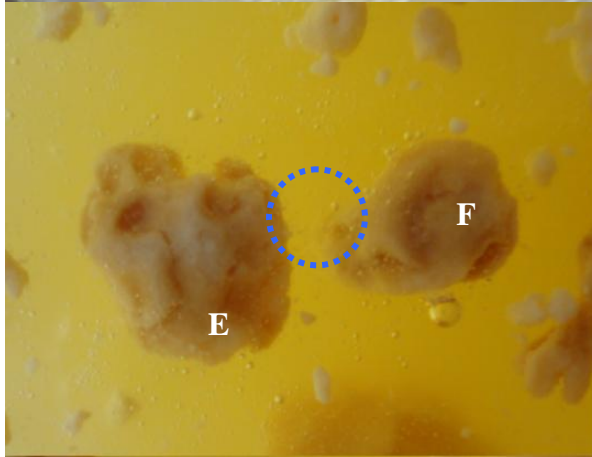
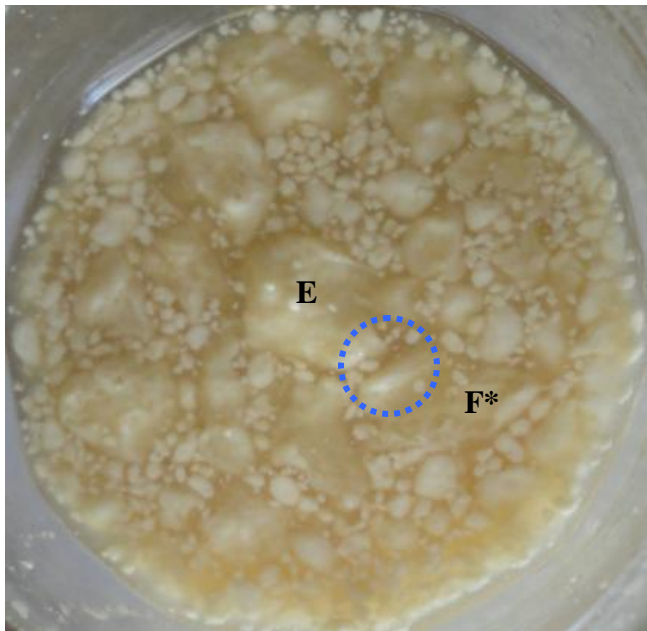




**Reference material (from image collection database)**



**Fig 12. Example of pair-bonding sustained during repeated cycles (Grains C and F).** There is an intimate connection visible with the naked eye (represented by circles). It is tempting to say that regardless of spatial location, some grains can locate other grains and interact specifically with them. The reader should refer to the reference material to aid in grain identification (also see additional video file). Close views show grain F upside down with an extended flap of biomass. Because of the complexity of this grain, top, bottom and side views are provided (labeled a, b, and c, respectively).



**Fig 13. Another example of pair-bonding sustained during repeated cycles (grains E and F).** The space in which the grains live has become an enormously complicated network. Regardless, both grains have a tendency to cluster together since the time they were originally discovered. They are not easily recognizable since they don't hold the same shape and have different orientation. There is an intimate connection visible with the naked eye (represented by circles). The weekly recurrence suggests that the grains did not interact randomly but are attracted to each other, even in crowded places. The grains have turned over, thereby exposing the other side (\*).